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(54) **Novel amyloid precursor proteins and methods of using same**

Neue Amyloid-Precursor-Proteine und Verfahren zur deren Verwendung

Nouveaux précurseurs de protéines amyloides et méthodes les utilisant

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(73) Proprietor: **AMERICAN CYANAMID COMPANY**
Wayne, NJ 07470-8426 (US)

(72) Inventors:
- **Vitek, Michael Peter**
  Waldwick, New Jersey 07463 (US)
- **Jacobsen, Jack Steven**
  Ramsey, New Jersey 07446 (US)

(74) Representative:
Wächtershäuser, Günter, Prof. Dr.
Patentanwalt,
Tal 29
80331 München (DE)

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- **SCIENCE vol. 248, 1990, pages 492 - 495 S.S. SISODIA ET AL.; 'Evidence that beta-amyloid protein in Alzheimer's Disease is not derived by normal processing'**
- **JOURNAL OF BIOLOGICAL CHEMISTRY vol. 267, 1992, pages 25602 - 25608 S.R. SAHASRABUDHE ET AL.; 'Release of amino-terminal fragments from amyloid precursor protein reporter and mutated derivatives in cultured cells'**

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Description

BACKGROUND OF THE INVENTION

[0001] Throughout this application various references are referred to within parentheses. Disclosures of these publications in their entirety are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citations for these references may be found at the end of this application, immediately preceding the claims.

[0002] Abnormal accumulation of extracellular amyloid in plaques and cerebrovascular deposits are characteristic in the brains of individuals suffering from Alzheimer's disease (AD) and Down's Syndrome (Glenner and Wong, BBRC, 120:885-890, 1984; Glenner & Wong, BBRC, 120:1131-1153, 1984). The amyloid deposited in these lesions, referred to as beta amyloid peptide (BAP), is a poorly soluble, self-aggregating, 39 to 43 amino acid (aa) protein which is derived via proteolytic cleavage from a larger amyloid precursor protein (APP) (Kang et al., Nature 325:733-736, 1987) BAP also is thought to be neurotoxic (Yankner et al., Science 245:417-420, 1990). APP is expressed as an integral transmembrane protein (Dyrks et al., EMBO. J., 7:949-957, 1989) and is normally proteolytically cleaved by "secretase" (Sisodia et al., Science, 248:492-495, 1990; Esch et al., Science, 248:1122-1124) between BAP-16K (lysine) and -17L (leucine). Cleavage at this site therefore precludes amyloidogenesis (Palmert et al., BBRC, 156:432-437, 1988) and results in release of the amino-terminal APP fragment which is secreted into tissue culture medium (Sisodia et al., ibid, Esch, et al., ibid). Three major isoforms of APP (APP-695, APP-751 and APP-770 amino acids) are derived by alternative splicing (Ponte, et al., Nature 331:525-527, 1988; Kitaguchi et al., Nature 331:530-532, 1988; and Tanzi, et al., Nature 331:528-530, 1988), are expressed as integral transmembrane proteins (Kang et al., Nature 325:733-736, 1987; Dyrks et al., EMBO J. 7:949-957, 1988).

[0003] Even though both APP-770 and -751 isoforms contain a protease inhibitor domain, it is the secreted portion of APP-751 (also known as Protease Nexin II (Van Nostrand et al., Science, 248:745-748, 1990) which is thought to be involved in cell adhesion (Schubert et al., Neuron, 3:689-694, 1989), remodeling during development, coagulation (Smith et al., Science, 248:1126-1128, 1990) and wound repair.

[0004] Although the mechanisms underlying abnormal proteolytic processes which result in BAP extraction from APP are poorly understood, it is thought to be central to the pathogenesis (Selkoe, Neuron, 6:487-498, 1991; Isiura, J. Neurochem. 56:363-369, 1991) and memory loss (Flood, et al., Proc. Natl. Acad. Sci. 88:3363-3366, 1991) associated with Alzheimer's Disease.

[0005] Based on the observations that (a) amyloid plaques develop in AD brains, (b) a major component of plaques is BAP, (c) BAP is generated by proteolytic cleavage of APP protein, (d) mRNA levels of specific APP isoforms increase in AD suggesting that more APP protein is expressed, (e) APP point mutations which are thought to possibly after normal processing have been identified in Familial AD (FAD) and "Dutch" disease, (f) injection of BAP into the brains of rodents both form lesions reminiscent of plaque pathology and result in memory deficits, and (g) the detection of plaque-like amyloid deposits in the brains of transgenic mice expressing human APP, it is important to understand how APP is processed to generate BAP.

SUMMARY OF THE INVENTION

[0006] This invention provides novel nucleic acid molecules which encode amyloid precursor muteins and the polypeptides encoded therefrom. Also provided are host vector systems useful for the recombinant production of the recombinant polypeptides in procaryotic and eucaryotic systems. Cells comprising the host vector systems of this invention as well as methods of recombinantly producing these polypeptides are provided by this invention. Further provided is a method to detect the recombinant polypeptides of this invention. Further provided is a method of screening for a compound which inhibits or augments the formation of β-amyloid protein.

BRIEF DESCRIPTION OF THE FIGURES

[0007] Figure 1: Schematic representation of APP-REP 751. APP-REP 751 represents a cleavable APP substrate system which contains target sequences of BAP including normal flanking regions (not to scale). The APP-REP protein is marked with a 276 amino acid deletion (corresponding to APP-751 beginning at XhoI through to and including the glycine codon at 15 amino acid residues N-terminal to BAP) and the insertion of sequences encoding N- and C- terminal reporter epitopes. Substrate P (SP) reporter epitope (RPKPQQFFGLM) is inserted at the XhoI site. Met-enkephaline (ME) reporter epitope (YGGFM) is inserted at the C-terminus of APP. The resulting construct encodes 492 amino acids (see Figure 2).

[0008] Figure 2: Schematic representation depicting the construction of APP-REP from APP-751 cDNA. Partial representing N- and C-terminal regions of APP-REP were cloned separately as illustrated below. The N-terminal partial

was constructed by ligating sequences encoding substance P (SP) to an N-terminal fragment of APP cDNA. The C-terminal partial was constructed by PCR amplification using the corresponding portion of APP cDNA to introduce novel ends including the Met-enkephalin (ME) reporter epitope. A functional APP-REP 751 clone was obtained by subcloning the partials as indicated. EcoRI (E), XhoI (X), HindIII (H), BamHI (B), SalI (S), XbaI (Xb).

[0009] Figure 3: Epitope mapping of APP-REP 751 expressed in COS-1 cells. Immunoprecipitation analysis of cell lysate and conditioned medium using the SP (anti-N-terminal substance P reporter) and M3 (anti-C-terminal APP) antisera. Lanes 1 and 2, cell lysate immunoprecipitated with SP and M3 antisera, respectively; lanes 3 and 4, conditioned medium immunoprecipitated with M3 and SP antisera, respectively; lanes 5 and 6, conditioned medium of control cells transfected with vector DNA immunoprecipitated with SP and M3 antisera, respectively; lane M, molecular weight markers.

[0010] Figure 4: Pulse-chase analysis of APP-REP 751. Immunoprecipitation of cell lysate (A) and CM (B). COS-1 cells were pulsed with [^{35}S]-methionine for 15 minutes and chased using cold methionine for 0, 0.5, 1, 1.5, 2 and 4 hours (lanes 1 to 6). Lanes 7, 8 and 9 are chase intervals of 0, 1 and 2 hour for control cells transfected with vector DNA. Lane M, molecular weight markers.

[0011] Figure 5: Epitope mapping and comparative expression of APP-REP 751, $BAP_{E22Q}$and $BAP_{\Delta}{}^{11-28}$.A, Schematic representation of relevant BAP (boxed) and flanking amino acid sequences of APP-REP 751, $BAP_{E22Q}$and $BAP_{\Delta 11-28}$juxtaposed against the putative transmembrane domain (shadowed). B-F, Immunoprecipitation analysis with antibodies recognizing indicated substance P (SP), KPI domain (KPI), C-terminal APP (M3) or Met-enkephalin (ME) epitopes; Lane M, molecular weight marker. B, Conditioned medium obtained from COS-1 cells expressing APP-REP 751 (lane 3), BAP_{E22Q}(lanes 4, 6 and 8), $BAP_{\Delta 11-28}$(lanes 5, 7 and 9) or control cells with (lane 2) or without (lane 1) transfection with vector DNA. C, Cell lysates obtained from COS-1 cells expressing APP-REP BAP_{E22Q}(lanes 1, 4 and 7), BAP_{Δ} 11-22 (lanes 2, 5 and 8) and control cells transfected with vector DNA (lanes 3, 6 and 9). D, Accumulation of secreted APP-REP 751 fragments in the conditioned medium obtained from COS-1 cells expressing APP-REP 751 (lanes 2 and 6), BAP_{E22Q} (lanes 3 and 8), $BAP_{\Delta 11-28}$(lanes 4 and 7), or control cells transfected with vector DNA (lanes 1 and 5), were pulsed with [^{35}S]-methionine and chased for 45 (lanes 1-4) or 90 (lanes 5-8) minutes with cold methionine. E, Accumulation of secreted APP-REP fragments in the conditioned medium obtained from stable (Chinese hamster ovary cells; lanes 1-4) and transient (COS-1 cells; lanes 5 and 6) expression of APP-REP 751 (lanes 2 and 5), $BAP_{\Delta 11-28}$ (lanes 3 and 6), BAP_{E22Q} (lane 4), or control cells transfected with vector DNA (lane 1).

[0012] Figure 6: Peptide mapping and sequencing of fragments secreted into the conditioned medium obtained from Chinese hamster ovary cells stably expressing APP-REP 751, BAP_{E22Q} and $BAP_{\Delta 11-28}$. A, Schematic representation depicting the APP-REP 751 and related derivative indicating the cleavage products and relevant carboxy-terminal fragments derived from treating the secreted fragments either with BNPS-Skatole (B) or cyanogen bromide. Downward- or upward-facing arrows represent BNPS-Skatole and cyanogen bromide cleavage sites, respectively. Amino acid lengths of relevant fragments for mapping or sequencing are given. B, BNPS-Skatole treatment of fragments secreted into the conditioned medium obtained from CHO cells stably expressing APP-REP 751 or $BAP_{\Delta 11-28}$. Mixture of conditioned medium containing APP-REP and $BAP_{\Delta 11-28}$(lane 1), or $BAP_{\Delta 11-28}$(lane 2) and APP-REP 751 (lane 3) alone.

[0013] Figure 7: Nucleotide and amino acid sequence of the APP-REP 751 protein.

[0014] Figure 8: Nucleotide sequence of APP 770 which also is available from the Genebank data base under accession number Y00264.

DETAILED DESCRIPTION OF THE INVENTION

[0015] This invention provides a nucleic acid molecule encoding an amyloid precursor mutein, wherein the nucleic acid molecule comprises, from the 5' end to the 3' end, a nucleic acid sequence encoding a marker and a nucleic acid sequence encoding the amino terminus of APP up to but not including the sequences that encode BAP. These nucleic acid molecules may include, but are not limited to the nucleic acid molecules selected from the group consisting of pCLL983, pCLL935, pCLL934 and pCLL913.

[0016] This invention also provides a nucleic acid molecule encoding an amyloid precursor mutein, wherein the nucleic acid molecule comprises, from the 5' end to the 3' end a nucleic acid sequence encoding BAP and a nucleic acid sequence encoding a marker. These nucleic acid molecules may include, but are not limited to the nucleic acid molecules selected from the group consisting of pCLL947, pCLL914, pCLL937, pCLL949 and pCLL957.

[0017] Further provided by this invention is a nucleic acid molecule which comprises the nucleic acid molecules defined hereinabove to each other. Method of ligating are well known to those of skill in the art. These nucleic acid molecules may include, but are not limited to the nucleic acid molecules selected from the group consisting of pCLL618, pCLL619, pCLL620, pCLL600, pCLL964, pCLL962, pCLL989, pCLL987, pCLL990, pCLL988, pCLL601, pCLL602, pCLL603, pCLL604, pCLL605, pCLL606 and pCLL607. These nucleic acid molecules are described in Table 3.

[0018] As used herein, the term "amyloid precursor mutein" is intended to encompass an amyloid precursor protein that is mutated, i.e., it is derived from a nucleic acid molecule which has changes in its primary structure as compared

to wild-type amyloid precursor protein (APP). Wild-type APP exists in three isoforms, thus, the nucleic acid molecule is changed in its primary structure for each of the three isoforms of wild-type APP. As is known to those of skill in the art, a mutation may be a substitution, deletion, or insertion of at least one nucleotide along the primary structure of the molecule. The mutations which are encompassed by this invention are the result of saturation mutagenesis in the regions of APP which are susceptible to cleavage by endoproteolytic enzymes. These mutations include deletions of nucleic acids encoding particular amino acids, substitution of nucleic acid sequences encoding one amino acid for a different amino acid and addition of nucleic acid sequences encoding additional amino acids not present in the wild type APP sequence. The term "marker" encompasses any substance capable of being detected or allowing the nucleic acid or polypeptide of this invention to be detected. Examples of markers are detectable proteins, such as enzymes or enzyme substrates and epitopes not naturally occurring in wild-type APP that are capable of forming a complex with an antibody, e.g. a polyclonal or monoclonal antibody. In the preferred embodiment of this invention, the marker is an epitope that is capable of being detected by a commercially available antibody. In one embodiment, the marker is an epitope capable of being detected by a monoclonal antibody directed to the Substance P, the Met-enkephalin or the c-myc epitope. In the most preferred embodiment of this invention, the marker is the c-myc epitopic region.

[0019] The term "BAP region" is defined as the region of APP wherein endoproteolytic cleavage will yield the amino-terminus and the carboxy-terminus of the BAP which is deposited as plaques and cerebrovascular amyloid in Alzheimer's disease brain. The function of the "BAP region" is to give rise to BAP which may function as a neurotoxic and/or neurotrophic agent in the brain and as other functionalities ascribed to BAP. The "BAP region" may also be endoproteolytically cleaved by enzymes. Such enzymes may include, but are not limited to the enzymes multicatalytic prtenase, propyl-endopeptidase, Cathepsin-B, Cathepsin-D, Cathepsin-L, Cathepsin-G or secretase. Secretase cleaves between lysine-16 (K-16) and leucine-17 (L-17) where full length BAP comprises the amino acid sequence DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA. Thus, for the purposes of this invention, the preferred embodiment is a cDNA which encodes an RNA which is translated into a protein which is the substrate for endoproteolytic activities which generate BAP.

[0020] In addition, for the purposes of this invention, the nucleic acid molecule may be DNA, cDNA or RNA. However, in the most preferred embodiment of this invention, the nucleic acid is a cDNA molecule.

[0021] This invention also encompasses each of the nucleic acid molecules described hereinabove inserted into a vector so that the nucleic acid molecule may be expressed, i.e., transcribed (when the molecule is DNA) and translated into a polypeptide in both procaryotic and eucaryotic expression systems. Suitable expression vectors useful for the practice of this invention include pSVL (Pharmacia), pRCRSV (Invitrogen), pBluescript SK^+ (Stratagene), pSL301 (Invitrogen), pUC19 (New England Biolabs). However, in the preferred embodiment of this invention, the vector pcDNA-1-neo is the expression vector for expression in eucaryotic cells. As is well known to those of skill in the art, the nucleic acid molecules of this invention may be operatively linked to a promoter of RNA transcription, as well as other regulatory sequences. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct the transcription of RNA off of the nucleic acid molecule. An example of a promoter is the human cytomegalovirus promoter. The vectors of this invention preferably are capable of transcribing and/or translating nucleic acid in vitro or in vivo. The recombinant polypeptides produced from the expression of the nucleic acid molecules of this invention are also provided.

[0022] A host vector system for the production of the recombinant polypeptides described hereinabove and for expressing the nucleic acid molecules of the subject invention are provided. The host vector system comprises one of the vectors described hereinabove in a suitable host. For the purpose of the invention, a suitable host may include, but is not limited to a eucaryotic cell, e.g., a mammalian cell, a yeast cell or an insect cell for baculovirus expression. Suitable mammalian cells may comprise, but are not limited to Chinese hamster ovary cells (CHO cells), African green monkey kidney COS-1 cells, and ATCC HTB14 (American Type Tissue Culture). Most preferably, the cell lines CRL 1650 and CRL 1793 are used. Each of these are available from the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, Maryland U.S.A. 20852. Suitable procaryotic cell may include, but are not limited to bacteria cells, HB101 (Invitrogen), MC1061/P3 (Invitrogen), CJ236 (Invitrogen) and JM109 (Invitrogen). Accordingly, the procaryotic or eucaryotic cell comprising the vector system of this invention is further provided by this invention.

[0023] As is known to those of skill in the art, recombinant DNA technology involves insertion of specific DNA sequences into a DNA vehicle (vector) to form a recombinant DNA molecule which is capable of being replicated in a host cell. Generally, but not necessarily, the inserted DNA sequence is foreign to the recipient DNA vehicle, i.e., the inserted DNA sequence and DNA vector are derived from organisms which do not exchange genetic information in nature, or the inserted DNA sequence comprises information which may be wholly or partially artificial. Several general methods have been developed which enable construction of recombinant DNA molecules. For example, U.S. Patent No. 4,237,224 to Cohen and Boyer describes production of such recombinant plasmids using processes of cleavage of DNA with restriction enzymes and joining the DNA pieces by known method of ligation.

[0024] These recombinant plasmids are then introduced by means of transformation or transfection and replicated in unicellular cultures including procaryotic organisms and eucaryotic organisms and eucaryotic cells grown in tissue

culture. Because of the general applicability of the techniques described therein, U.S. Patent No. 4,237,224 is hereby incorporated by reference into the present specification. Another method for introducing recombinant DNA molecules into unicellular organisms is described by Collins and Hohn in U.S. Patent No. 4,304,863 which is also incorporated herein by reference. This method utilized a packaging, transduction system with bacteriophage vectors (cosmids).

[0025] Nucleic acid sequences may also be inserted into viruses, for example, a vaccinia virus or a baculovirus. Such recombinant viruses may be generate, for example, by transfection of plasmids into cells infected with virus, Chakrabarti et al, (1985) Mol. Cell Biol. 5:3402-3409.

[0026] Regardless of the method used for construction, the recombinant DNA molecule is preferably compatible with the host cell, i.e., capable of being replicated in the host cell either as part of the host chromosomes or as an extra-chromosomal element. The recombinant DNA molecule or recombinant virus preferably has a marker function which allows the selection of the desired recombinant DNA molecule(s) or virus, e.g., baculovirus. In addition, if all of the proper replication, transcription and translation signals are correctly arranged on the recombinant DNA molecule, the foreign gene will be properly expressed in the transformed or transfected host cells.

[0027] Different genetic signals and processing events control gene expression at different levels. For instance, DNA transcription is one level, and messenger RNA (mRNA) translation is another. Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes RNA synthesis. The DNA sequences of eucaryotic promoters differ from those of procaryotic promoters. Furthermore, eucaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a procaryotic system.

[0028] Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno (SD) sequence on the mRNA. For a review on maximizing gene expression, see Roberts and Lauer (1979) Methods in Enzymology 68:473.

[0029] Many other factors complicate the expression of foreign genes in procaryotes even after the proper signals are inserted and appropriately positioned. One such factor is the presence of an active proteolytic system in E. coli and other bacteria. This protein-degrading system appears to destroy foreign proteins selectively. A tremendous utility, therefore, would be afforded by the development of a means to protect eucaryotic proteins expressed in bacteria from proteolytic degradation. One strategy is to construct hybrid genes in which the foreign sequence is ligated in phase (i. e., in the correct reading frame) with a procaryotic structural gene.

[0030] Expression of this hybrid gene results in a recombinant protein product (a protein that is a hybrid of procaryotic and foreign amino acid sequences).

[0031] Successful expression of a cloned gene requires efficient transcription of DNA, translation of the mRNA and in some instances post-translation modification of the protein. Expression vectors have been developed to increase protein production from the cloned gene. In expression vectors, the cloned gene is often placed next to a strong promoter which is controllable so that transcription can be turned on when necessary. Cells can be grown to a high density and then the promoter can be induced to increase the number of transcripts. These, if efficiently translated, will result in high yields of polypeptide. This is an especially valuable system if the foreign protein is deleterious to the host cell.

[0032] Several recombinant DNA expression systems are described below in the Experimental Procedures section for the purpose of illustration only, and these examples should not be construed to limit the scope of the present invention.

[0033] A method for producing a recombinant polypeptide described hereinabove, is also provided. This method comprises growing the host cell containing the nucleic acid of this invention and/or the host vector system of this invention under suitable conditions, permitting production of the polypeptide and recovering the resulting recombinant polypeptide produced.

[0034] A method of detecting in a sample the presence of any of the recombinant polypeptides described hereinabove is further provided by this invention. In the preferred embodiment of this invention, the marker is an epitope directed against an antibody, the epitope of which is not present in the wild-type polypeptide or APP derivative. This method comprises obtaining a sample suspected of containing the polypeptide and contacting the sample with an antibody directed to the marker. The contacting is done under suitable conditions to favor the formation of an antibody-epitope (i.e., antigen) complex, and detecting the presence of any complex so formed. The presence of complex being a positive indication that the recombinant polypeptide is in the sample. In one embodiment of this invention, the antibody is a mouse antibody. In another embodiment of this invention, the antibody is a human antibody. In the most preferred embodiment, the mouse or human antibody is either a mouse or human monoclonal antibody.

[0035] The antibody is labeled with a detectable marker selected from the group consisting of radioisotopes, dyes, enzymes and biotin. For the purposes of this invention, suitable radioisotopes include, but are not limited to, ^{32}P, ^{35}S, ^{131}I and ^{125}I.

[0036] Suitable samples for the practice of this invention include, but are not limited to conditioned media, cell lysates and cellular organelle fractions.

[0037] The method of this invention may utilize the recombinant polypeptide for the detection of drugs or compounds that inhibit or augment the activity of proteolytic enzymes which cleave APP to generate BAP fragments. For the purposes of example only, a recombinant polypeptide which contains a Substance-P marker epitope on the amino-terminal side of BAP and a Met-enkephalin marker epitope on the carboxy-terminal side of BAP. Using commercially available RIA kits (Peninsula), one can measure the amount of amino-marker and carboxy-marker in any given sample. Since endoproteolytic activity is shown (see Figure 3) to allow the release of amino-terminal fragments of APP containing the amino-marker into the conditioned media while carboxy-terminal APP fragments containing the carboxy-marker remain associated with the cell, then RIA measures the amount of amino-marker in the conditioned medium as a direct result of endoproteolytic cleavage activity between the marker epitopes preferably within the "BAP region". Using this RIA to the amino-marker, the effect of potential drugs designed to modify endoprotease activity can be tested comparing the level of amino-marker in untreated and endoprotease-inhibitor treated samples. If a difference in non-treated and treated samples is found, then the position of the cleavage or lack of cleavage can be verified as with the procedures used in Figures 3 to 6. Thus, the qualitative and quantitative aspects of endoproteolytic activity and its inhibition on the recombinant APP mutein is evaluated. The amino-marker also is an enzyme such as beta-galactosidase which would be released in the conditioned media by the action of an appropriate endoprotease. Cell free samples of conditioned media containing the liberated enzyme converts a chromogenic substrate into the appropriately colored product (Blue for X-gal and Yellow for ONPG) which is measured spectrophotometrically. Inhibitors of the appropriate endoprotease would inhibit the release of beta-galactosidase enzyme into the conditioned medium resulting in less colored product being observed.

[0038] It is a purpose of this invention to develop a cleavable APP substrate system which represents target sequences of BAP including normal flanking regions to provide recognition sequences for processing enzymes. The utilization of a common substrate for parallel strategies involving in vitro cleavage assays using cellular extracts, in vivo processing assays in tissue culture or bacterial cells, or in conjunction with a selection system aimed at cloning BAP-cleaving proteases (or other relevant proteins) is preferred.

[0039] A second purpose of this invention is to develop an APP substrate which is non-cleavable by secretase in order to better detect other putative abnormal processing events which are hypothesized to potentially either compete with secretase for limited substrate, or occur at much lower frequency than secretase and whose effects may be otherwise masked by the mass action of secretase. These are referred to as "secretase-minus mutants" in Table 4.

[0040] Third, secretase-cleavable and -noncleavable APP substrates would provide probes with which to investigate cellular post-translational modifications to APP in an attempt to determine the potential influence on normal secretase and abnormal BAP "clipping" activities. These areas include, among others, the consideration of various known APP point mutations, contribution by different cell/tissue types (normal- or AD-specific), the Kunitz Protease Inhibitor domain present in APP-770 and - 751 isoforms, APP phosphorylation and APP glycosylation.

[0041] These are referred to as "APP 717 mutations" or Dutch Disease Mutations in Table 5.

[0042] Fourth, the ability to detect specific APP proteolytic events, either the normal secretase or the abnormal BAP-generating activities, would enable the use of strategies which use phenotypic rescue as a marker for the cloning of potentially relevant and interesting proteases in tissue culture systems.

Overview of the APP-REP Strategy

[0043] To study secretase and BAP-generating pathways, portions of APP cDNA clones are used to engineer a panel of APP-REPorter (APP-REP) plasmids to express "marked" proteins representing each of the APP isoforms (and other APP/BAP sequence alterations; see below) in cultured cells. The system utilizes the marker Substance-P (SP) and Met-Enkephalin (ME) which are strategically placed, respectively, on amino- and carboxy-terminal sides of BAP. Proteolytic cleavage of APP-REP target substrate is determined by the electrophoretic sizing of resulting proteolytic fragments and immunological detection of APP-specific and SP and ME reporter epitopes. Deletion of a large central portion of APP sequence also makes APP-REP readily distinguishable from the endogenous APP isoforms based on size. Moreover, the resolution of detecting proteolytic cleavage at different positions within the APP-REP substrate protein is enhanced by working with shorter target substrates. Approximate location of cleavage is determined initially by fragment sizing and epitope mapping; the exact cleavage site is later determined by peptide mapping of affinity/HPLC purified fragments and sequencing of peptide ends.

[0044] Plasmids also are derived from these constructs for developing similar strategies to express APP-REP protein in cell free reticulocyte transcription-translation and bacterial systems. Mutation of APP-REP secretase/BAPase cleavage site (by sequence substitution, deletion or FAD mutations) can reveal putative proteolytic activities associated with BAP formation including amino- and carboxy-BAPase activities which are predicted to result in altered product fragments lengths.

FIRST SERIES OF EXPERIMENTS

Bacterial Strains and Transformation

[0045] Transformation of commercially available frozen competent bacteria, maintenance and selection of transformants is according to the manufacturer. Strains HB101, DH5a or JM109 (Gibco-BRL) are used for the construction of APP-REP in pSK(+) (Stratagene, La Jolla, CA) and pSL 301 (Invitrogen, San Diego, CA). APP-REP is subsequently subcloned into the eucaryotic expression vector pcDNA-1-neo and amplified in MC1061/P3 (Invitrogen, San Diego, CA).

Plasmid Construction

[0046] A cassette approach is used to independently construct portions of the APP-REP plasmid (Figure 2). The N-terminal partial includes APP sequences through the Substance P (SP) epitope, while the carboxy-terminal (C-terminal) partial includes BAP (or sequence variations of BAP) through the Met-enkephalin (ME) epitope (Figure 1). A plasmid encoding the N-terminal cassette (either of the plasmids listed in Table 1A, which include pCLL983, pCLL935, pCLL934 and pCLL913) is constructed by ligating the EcoRI-XhoI fragment derived from each of the corresponding APP cDNAs listed in Table 1A,which include APP-695, APP-751 or two different APP-770 sequences,to a short synthetic XhoI-HindIII fragment encoding Substance P (amino acid 1-11). This product is then ligated into the EcoRI and HindIII sites of pSK(+). Plasmid encoding the carboxy-terminal (C-terminal) cassette (pCLL947) is constructed by cloning into the HindIII-BamHI sites of pSL301 a fragment containing BAP sequences which is amplified by polymerase chain reaction.

[0047] The fragments feature a novel 5'-HindIII site beginning at lysine 638 of APP-751, native or modified BAP sequences, and additional full-length or truncated APP sequences. The C-terminal cassette provides APP C-terminal wild type sequences, truncations following the transmembrane domain of BAP sequence, an E to Q substitute at BAP as#22, or a G to A substitute at BAP aa#10 with the deletion of aa#11-28 and creation of a novel NdeI site. Each of the APP C-terminal variantes contain the additional Met-enkephalin sequences. Each of the resulting pSL301 HindIII-SalI fragments (including HindIII-BamHI coding region plus BAmHI-SalI polylinker sequences) is then isolated and ligated pairwise with each of the N-terminal cassettes by subcloning into the HindIII-SalI sites of the SK(+)-based plasmid to generate the panel of new plasmids identified in Table 2. Next, the polylinker of the CMV promotor driven eukaryotic expression vector, pcDNA-1-neo (pCLL601), was modified to accommodate the panel of plasmids listed in Table 2 of XbaI-SalI APP and APP-Rep fragments to create a second panel of plasmids listed in Table 3 for eukaryotic expression.

[0048] Polylinker modification involves the substitution of the HindIII-XbaI fragment with a synthetic one which restores HindIII, destroys XbaI and introduces novel BamHI-XabI-Xho-SalI sites.

Tissue Culture Lines

[0049] All cells are obtained from American Type Culture Collection and maintained according to their recommendation. They include SV40-transformed African Green monkey kidney COS-1 cells (CRL 1650) for transient expression and Chinese hamster ovary CHO-1C6 (CRL 1973) for stable expression systems.

Transfection Procedure

[0050] Cells are seeded at a density of 2-3 X 10^6/100 mm dish and transfected using Lipofectin (Gibco-BRL, Grand Island, NY) when ~75% confluent. Plasmid DNA (0.5-4 mg) is diluted in 450 ml of Opti-MEM (Gibco-BRL, Grand Island, NY), mixed with 450 ml containing 75-100 ml Lipofectin and the mixture incubated at room temperature for 20-30 minutes. Addition of DNA-Lipofectin mixture to cells, recovery phase and G418 selection (Gibco-BRL), when applicable, are according to the manufacturer's protocol. Cells and conditioned medium are harvested at 48-72 hours following transfection for assay of APP-REP expression.

Antisera

[0051] APP-specific antisera:anti-N-terminal APP, mouse monoclonal 22C11 (Boehringer-Mannheim Biochemicals, Indianapolis, IN) raised against a recombinant fusion protein expressing APP-695 (epitope mapped to aa 60-100); anti-KPI rabbit polyclonal, raised against recombinant protein encoded by the HinfI fragment derived from APP-770; and anti-APP C-terminal rabbit polyclonal M3, raised against synthetic APP peptides corresponding to APP-770 amino acid residues 649-671. Reporter-specific antisera:anti-substance P, rabbit polyclonal, purchased from Peninsula, Belmont, CA; and anti-Met-enkephalin, rabbit polyclonal, purchased from Cambridge, Wilmington, DE.

[0052] Preparation of Radiolabeled APP-REP and Extraction from Conditioned Medium and Cell Lysates

[0053] APP-REP proteins transiently expressed in exponentially growing adherent cells (~4 x 10^6) are radiolabeled by metabolic incorporation of [^{35}S]-methionine as follows. Cell monolayers are washed twice with prelabeling medium (methionine-free D-MEM supplemented with glutamine, sodium pyruvate, antibiotics and 1% dialyzed fetal bovine serum (Gibco-BRL) and incubated for 15 minutes to 4 hours in prelabeling medium containing 150-450 uci[^{35}S]-methionine (Amersham, 800Ci/mmol). If chased with cold methionine, the medium is removed following the pulse, the monolayer is washed with prelabeling medium and replaced with 3 ml of the same containing 1 mM cold methionine.

[0054] The conditioned medium is recovered following radiolabeling by aspiration from plates and cell debris removed by centrifugation for 10 minutes at 4°C (-300xg). Conditioned medium is immediately supplemented with protease inhibitors (pepstatin A, 50 ug/ml; leupeptin, 50 ug/ml; aprotinin, 10 ug/ml; EDTA, 5 mM; PMSF, 0.25 mM) and immunoprecipitation buffer (IPB; Sisodia et al., 1990) for protein analysis. Briefly, 3 ml of CM is supplemented with 0.75 ml 5X IPB (250 mM Tris, pH 6.8; 750 mM NaCl; 25 mM EDTA; 2.5% Nonidet P40; 2.5% sodium deoxycholate) and incubated for 20 minutes at 4°C prior to use.

[0055] Lysates are prepared by washing the labeled cell monolayer twice with 5 ml pre-labeling medium and directly extracting cells in plates at 4° C with 3.75 ml 1X IPB (including protease inhibitors). Cells are scraped into the buffer, incubated for 20 minutes at 4°C and lysates clarified of cellular debris by centrifugation for 20 minutes at 10,000xg.

[0056] For radioiodination of cell surface proteins, monolayers are chilled on ice, washed 3 times with 5 ml ice cold PBS and labeled at room temperature for 10 minutes following the addition of: 5 ml PBS containing 0.2 mCi Iodine-125 (NEZ-033A, New England Nuclear), 0.25 ml lactoperoxidase (1 mg/ml distilled water, Sigma), 10 ul of hydrogen peroxide solution (freshly prepared by diluting 10 ml of 30% stock in 10 ml of PBS) added at 0, 3, 6, and 9 minutes of iodination. At 10 minutes, the supernatant is removed and cells gently washed with 10 ml of ice cold PBS (containing 10 mM NaI). Four ml of PBS is added, and CM and cell lysates are prepared as above.

Immunoprecipitation Analysis

[0057] Aliquots of radiolabeled lysate or conditioned medium representing 4-8x10^5 cells are thawed on ice, supplemented with protease inhibitors (see above), boiled for 3 minutes in 0.35% SDS and chilled on ice. Samples are preincubated on a shaker for 1.5 hours at 4°C with 2-10 ul 2X of preimmune (or normal rabbit) serum and 2 mg Protein A-Sepharose (Sigma; prepared in 1X IPB), and insoluble immune removed by centrifugation. APP-or reporter epitope-specific antisera (0.1-10 ul) and 2 mg Protein A-Sepharose were similarly added and incubated overnight. Specific immune complexes were precipitated, washed 4 times with 0.25 ml 1 X IPB (with protease inhibitors), extracted with 20 ul Laemmli sample buffer (Laemmli (1970) Nature 227:680-685), boiled for 3 minutes and fractionated by electrophoresis on SDS-polyacrylamide-tris-glycine (Bio-Rad Laboratories, Richmond, VA) or SDS-polyacrylamide-tris-tricine Daiichi (Integrated Separation Systems, Natick, MA) gels. Gels are then treated with Enlightening Autoradiographic Enhancer (New England Nuclear, NEF-974) and dried in vacuo with heat and exposed to Kodak X-AR film at -70°C.

Western (Immunoblot) Analysis

[0058] Lysate or 10X concentrated conditioned medium (Centricon 30 microconcentrator; Amicon, Beverly, MA) representing 4-8x10^5 cells are supplemented with an equal volume of 2X Laemmli sample buffer, boiled for 2 minutes, fractionated by electrophoresis on SDS-polyacrylamide-tris-glycine (Bio-Rad, XX) or SDS-polyacrylamide-tris-tricine Daiichi (Integrated Separation Systems, Natick, MA) gels and transblotted (Semi-Phor, Hoefer Instruments, San Francisco, CA) to Immobilon-P membrane (Millipore, Bedford, MA). Membranes are pre-blocked in 10 ml 5% non-fat dry milk/PBST (PBS with 0.02% Tween-20) for 45 minutes at room temperature prior to overnight incubation at 4°C with primary antisera (in fresh pre-blocking solution). Blots are then washed, incubated with secondary antibody, washed and developed for horseradish peroxidase activity as described (ECL Luminol Kit; Amersham, Arlington Heights, IL).

Peptide Mapping and Determination of the Site of Proteolytic Cleavage by Peptide Sequencing

[0059] The secretase clip site is determined essentially as described (Wang et al., (1991) J. Biol. Chem. 266: 16960-16964). Approximately 1X10^6 CHO cells stably expressing APP-REP are seeded in each 150 mm dish containing DMEM (complete with 200 ug/ml G418) and incubated for 36 hours. Cells are washed, preincubated for 6 hours in serum-free medium (MCDB 302 supplemented with antibiotics, L-glutamine (292 mg/l) and proline 12 mg/l (Sigma) to remove serum components, washed, and incubated for another 72 hours in fresh serum-free media.

[0060] Serum-free conditioned medium was pooled and cell debris was removed by centrifugation (10 minutes at 300xg, then 30 minutes at 100,000xg) and concentrated by acetone precipitation and fractionated by FPLC. Conditioned medium concentrate is loaded on an anion exchange column (Mono Q; source) and protein is eluted in 20 mM Tris (pH 7.4) over a 0-1M NaCl gradient. Fractions containing secreted APP are identified by immunoblotting (mono-

clonal antibody 22C11) and relevant samples pooled, desalted (NP-5 column; Pharmacia, Piscataway, NJ) and concentrated. Proteins are then denatured, treated with cyanogen bromide (in 10% trifluoroacetic acid) and peptides separated by high performance liquid chromatography (Vydac C_{18} reverse-phase) attached to a FAB-MS unit. Relevant peaks derived from APP-REP 751 and APP-REP BAP $_{11-28}$are identified by locating these peaks uncommon to both proteins. The C-terminal peptides derived from APP-REP BAP $_{11-28}$(predicted 14 amino acid) and APP-REP 751 (predicted 17 amino acid) are sequenced (MilliGen solid phase peptide sequencer; Millipore, Burlington, MA).

EXPERIMENTAL RESULTS

Characterization of APP-REP Expression by Epitope Mapping

[0061] The APP-REP strategy (Figure 1) is a system for the expression of marked APP proteins in tissue culture cells in order to characterize the proteolytic cleavage events. The deletion of a 276 amino acid portion distinguishes the construct of this invention from endogenously expressed APP on the basis of size, and is predicted to increase the resolution of APP-REP fragments resulting from the proteolytic cleavage by secretase or other amyloidogenic, BAP-generating cleavage events. Substance P and Met-enkephalin marker epitopes strategically placed on either side of BAP enable the immunological detection of N- and C-terminal fragments, respectively, which result from proteolytic cleavage of APP-REP substrate.

[0062] APP-REP protein transiently expressed in COS-1 cells has been radiolabeled by metabolic incorporation of [^{35}S]-methionine in a 60 minute pulse, immunoprecipitated with antisera, and size fractionated by gel electrophoresis as demonstrated in Figure 3. Immunoprecipitation with a panel of APP- and APP-REP-specific antisera which recognize epitopes mapping at various positions along APP-REP, reveals the presence of 2 proteins of ~63 kDa in cell lysates (including cytoplasmic and membrane associated proteins) as shown in Figure 3. The specific detection by antisera directed against the KPI domain, the carboxy-terminus of APP (M3, Figure 3A) and Met-enkephalin, as well as by the N-terminal 22C11 monoclonal in Western blot analysis (data not shown), suggests that both bands represent the full-length APP-REP protein. Although the 492 amino acid APP-REP is predicted to display a mobility of -49-54 KdA, the larger 63 and 76 kDa proteins are expected based on previous observations attributing the aberrant migration properties of APP, putatively to post-translational modification like tyrosine-sulfation, glycosylation and phosphorylation (Dyrks et al., (1988) EMBO J. 7:949-957; Weidemann et al., (1989) Cell 57:115-126.

[0063] Analysis of the conditioned medium (CM) collected from those same cells above indicates that an N-terminal fragment of APP-REP is released into the CM. Figure 3B reveals a shorter -67 kDa fragment immunoprecipitable from CM with KPI and SP antisera (and the 22C11 monoclonal by Western analysis), but not with several C-terminal APP or ME antisera. These data are consistent with the observations (Selkoe et al., (1988) P.N.A.S. 86:6338-6342; Palmert et al., (1989 a) P.N.A.S. U.S.A. 85:7341-7345), b) indicating that APP is a substrate for the proteolytic cleavage resulting in the secretion of an N-terminal fragment into CM, and a short membrane associated C-terminal fragment.

Pulse-Chase Analysis Reveals the Precursor/Product Relationship Between Cell Associated and Secreted Derivatives of APP-REP

[0064] To show that APP-REP undergoes post-translational modification accounting for the 2 cell associated proteins, and that the N-terminal APP-REP fragment released into CM is derived from one of these precursors, radiolabeled APP-REP is with a short 15 minute pulse and collected both cell lysates and CM at various chase intervals as shown in Figure 4. Immunoprecipitation analysis reveals that APP-REP initially migrates at -63 kDa and is rapidly "chased" up to ~76 kDa with conversion rate of less than 10-15 minutes (Figure 4A; also see Figure 5C for quantitative analysis), an observation which is consistent with the notion that APP-REP, like APP, is a substrate for post-translational modifications.

[0065] The -76 kDa APP-REP band (cell lysate) rapidly disappears (t $^{1/2}$ -20 minutes) (Figure 4A and 5C), followed by the appearance of a shorter -67 kDa band in the CM (Figure 4B and 5C). The released -67 kDa fragment accumulates rapidly and is relatively long lived (t 1/2 > 8 hours). The temporal pattern of intracellular APP-REP depletion, accumulation of a shorter ~67 kDa protein in CM, and the recognition of this protein only by antisera raised against N-terminal epitopes, is consistent with proteolytic cleavage of APP-REP which is similar to the normal, non-amyloidogenic, "secretase" activity which results in the release of an N-terminal APP fragment (Sisodia et al., (1990) Science 248:492-495.

Expression of APP-REP Derivatives Containing Altered BAP Sequences Does Not Prevent Proteolytic Cleavage

[0066] In an attempt to engineer non-cleavable substrates for secretase, APP-REP proteins are expressed (Figure 5A) either lacking the secretase "cleavage/recognition site" putatively encompassed by aa residues BAP 11-28 (BAP$_{\Delta 11-28}$pCLL604), or representing the BAP point mutation found in patients with HCHWA-D (BAP $_{E22Q}$,pCLL603).

The construct representing the BAPE22Q mutation results in secretion of an N-terminal fragment indistinguishable from the APP-REP protein (Figure 5C). Deletion of extracellular, juxtamembranous 18 aa (BAP_{Δ} 11-28), however, still results in the secretion of an N-terminal APP-REP fragment into the CM (Figure 5B). A slightly faster migration of fragment derived from the deletion construct pCLL604 in comparison to that of wild-type pCLL602, is consistent with the 18 aa deletion and a corresponding loss of ~2 kDa (Figure 5C). Pulse-chase analyses (Figure 5D) indicate that expression of full-length precursor by each construct, proteolytic cleavable and the release of fragment into CM is both qualitatively and quantitatively similar to that of the wild-type APP-REP sequence. Chinese hamster ovary (CHO) cells stably expressing APP-REP display results similar to that of transiently expressing COS-1 cells (Figure 5E). Collectively, these data suggest that the cleavage in each case may be the result of similar biochemical events despite the difference in juxtamembranous sequences (Figure 5A).

Full-Length APP-REP Proteins Are Associated with Plasma Membrane Prior to Cleavage

[0067] In preliminary experiments, detection of the amino-terminal APP-REP fragment in CM and not in cell lysates, suggests that the putative secretase activity might be plasma membrane-associated. One prediction of this notion is that an N-terminal portion of APP-REP might be (partially) localized to the extracellular environment prior to cleavage. In order to test this hypothesis, CHO cells stably expressing APP-REP (pCLL602) are subjected to lactoperoxidase-catalyzed iodination to radiolabel only extracellular proteins associated with the cell surface, and CM and cell lysates were analyzed immediately following iodination or after a 10 minute incubation. Presence of the ~76 kDa APP-REP band in cell lysate should indicate that at least a portion of full-length APP-REP is poised extracellularly in association with cell membrane. Detection of both, a reduced fraction of the ~76 kDa band in the cell lysate and a corresponding increased fraction of ~67 kDa fragment in CM following the "release" incubation, would suggest that the extracellular portion of APP-REP is cleaved.

Peptide Sequencing to Determine the Site of Proteolysis

[0068] Fragment secreted into serum-free media derived from CHO cells stably expressing APP-REP with wild-type or BAP 11-28 sequences has been analyzed to determine the actual site of proteolytic cleavage as shown in Figure 6. Peptide mapping by tryptophan-specific cleavage with BNPS-skatole is used to roughly determine the approximate position of cleavage in each molecule. Western blot analysis using SP antisera following BNPS-skatole treatment (Figure 6B) reveals fragments whose lengths of -10.5 and -9.5 kDa, corresponding to wild type and BAP_{11-28}respectively, confirming that cleavage occurs in the C-terminal portion of the PN-2-like protein as expected (Figure 6A). To determine the actual position of cleavage, secreted fragment is partially purified, treated with cyanogen bromide and relevant C-terminal peptides derived from APP-REP wild type.

DISCUSSION

[0069] The expression of a truncated form of APP-751, namely APP-REP 751 (pCLL602) is examined and its normal cleavage by secretase. Comparison of the nontransfected cells and those transfected with APP-REP 751, in both COS-1 transient and CHO stable expression systems, show the production of shorter secreted protein derived from APP-REP. Furthermore, upon a prolonged exposure of the fluorogram only one band is observed in condition medium. Epitope mapping with antibodies to N- and C-terminal domains of APP-REP and amino acid sequencing suggest post-translational cleavage at a site similar to that reported for intact APP protein and other truncated APP constructs similar to that reported for intact APP protein and other truncated APP constructs. Pulse-chase experiments reveal post-translational modifications, believed to be similar to those described for the intact APP protein, in which a single ~63 kDa product is chased up to ~76 kDa in the first 30 minutes. Appearance of the ~76 kDa cell membrane associated protein precedes the release of a ~67 kDa product into the CM. The released form, which is not observed in the cell lysate fraction, steadily accumulates in the conditioned medium well after the ~76 kDa band has begun to disappear suggesting a precursor-product relationship. These data indicate that the APP-REP protein is a good representation of the naturally occurring APP with respect to post-translational synthesis, processing, and stability in a tissue culture system.

[0070] Epitope mapping of APP-REP 751 mutants suggest that BAP_{E22Q}, as well as the $BAP_{\Delta 11-28}$deletion constructs, are initially expressed as larger proteins of predicted lengths which subsequently are cleaved to release N-terminal fragments into the CM. The pulse-chase experiments indicate the cell-associated and secreted forms accumulate with similar kinetics.

TABLE 1

Construction of APP-REP Partials

A. pSK(+) Amino-Terminal Constructs: Cloning of APP Isoform and Reporter Epitope (EcoRI-HindIII Fragments)

Plasmid Name	APP Isoform (EcoRI-XhoI Fragment)	Reporter Epitope (XhoI-HindIII Fragment)
pCLL983	APP-695	Substance P*
pCLL935	APP-751	Substance P
pCLL934	APP-770**	Substance P
pCLL913	APP-770#	Substance P

Notes:

* Substance P is a peptide containing 11 residues with the amino acid sequence of RPKPQQFFGLM.

** 5' untranslated sequences derived from the shorter APP-770 cDNA form.

\# 5' untranslated sequences derived from the longer APP-751 cDNA form.

B. pSL301 Carboxy-Terminal Constructs: Cloning of BAP-Encoding APP Reporter Epitope Fusions (HindIII-BamHI/SalI Fragment)

Plasmid Name	Met-Enkephalin (ME) Fusion at end of:	Name of Variation
pCLL947	Full-Length APP	APP-BAP-APP-ME
pCLL914	Transmembrane Domain	APP-BAP-TM-ME
pCLL937	BAP	APP-BAP-ME

TABLE 1

Construction of APP-REP Partials

(Continued)

C. pSL301 Carboxy-Terminal Full-Length APP-ME Constructs: Introduction of Mutations in BAP (HindIII-BamHI/SalI Fragment)

Plasmid Name	Met-Enkephalin Fusion at End of:	Name of Variation
pCLL949	E to Q substitution at BAP aa#22	BAP E22Q
pCLL957	G to A substitution at BTaa#10, deletion of BAP AA#11-28 and creation of NdeI site	BAP-vaa11-28

TABLE 2

Assembly of APP-REP Full-Length Constructs Containing Substance P and Met-Enkephalin Reporter Epitopes and BAP or a Variation of BAP

Plasmid Name	Construct Name/Variation	Plasmid (N-Terminus)	Restriction Fragment (C-Terminus)
pCLL618	APP-REP-695	pCLL983	pCLL947
pCLL964	APP-REP-751	pCLL935	pCLL947
pCLL962	APP-REP-770	pCLL934	pCLL947
pCLL619	APP-REP-695/BAP_E to Q	pCLL983	pCLL949
pCLL989	APP-REP-751/BAP_E to Q	pCLL935	pCLL949
pCLL987	APP-REP-770/BAP_E to Q	pCLL934	pCLL949
pCLL620	APP-REP-695/$BAP_{\Delta aa11-28}$	pCLL983	pCLL957
pCLL990	APP-REP-751/$BAP_{\Delta aa11-28}$	pCLL935	pCLL957
pCLL988	APP-REP-770/$BAP_{\Delta aa11-28}$	pCLL934	pCLL957

TABLE 3
Subcloning of APP-REP Full-Length Constructs
and Human Growth Hormone (hGH) into pcDNA-1-Neo[XS]

Plasmid Name	Construct Name (in pcDNA-1-neo)	Source of Insert
pCLL600	pcDNA-1-neo-hGH	p0GH*
pCLL601	pcDNA-1-neo[XS]	Synthetic Fragment**
pCLL602	APP-REP-751	pCLL964
pCLL603#	APP-REP-751/$BAP_{E\ to\ Q}$	pCLL989
pCLL604#	APP-REP-751/$BAP_{\Delta aa11-28}$	pCLL990
pCLL605	APP-REP-770	pCLL962
pCLL606	APP-REP-770/$BAP_{E\ to\ Q}$	pCLL987
pCLL607	APP-REP-770/$BAP_{\Delta aa11-28}$	pCLL988

Notes:

* The HindIII-EcoRI (blunt-ended) fragment encoding hGH sequences of p0HG (Nichols Diagnostics) was subcloned into the HindIII-EcoRI (blunt-ended) sites of pcDNA-1-neo.

** The HindIII-XbaI fragment of the pcDNA-1-neo polylinker was replaced with a synthetic fragment which destroyed the original XbaI site and introduced several unique sites (HindIII-BamHI-XbaI-XhoI-SalI).

\# Also created by an alternative strategy using the same pSK(+) plasmids.

TABLE 4
"Secretase-Minus" APP-REP Constructs
Engineered by Oligonucleotide-Directed Mutagenesis

Plasmid Name	Mutation Identity	Mutated BAP Sequence Compared to Wild Type							Percent** Secretion	
		14	15	16	17	18	19	20		
pCLL602	BAP*	CAT	CAA	AAA	TTG	GTG	TTC	TTT	100	31844 E
		H	Q	K	L	V	F	F		
pCLL608	BAP-16KE	CAT	CAA	GAG	TTG	GTG	TTC	TTT	0	31844 F
		H	Q	E	L	V	F	F		
pCLL609	BAP-16KV	CAT	CAA	GTG	TTG	GTG	TTC	TTT	10-20	31844 G
		H	Q	V	L	V	F	F		
pCLL610	BAP-19FP	CAT	CAA	AAA	TTG	GTG	CCG	TTT	10-20	31844 H
		H	Q	K	L	V	P	F		

Notes:

* Wild-type BAP

* % secretion relative to wild type BAP sequence as determined by Sisodia.

TABLE 5
APP-REP Constructs Modeling APP Mutations
Associated with Diseases Involving BAP Deposition

APP "717" MUTATIONS

		// APP Transmembrane Domain / // [BAP]								
		711	712	713	714	715	716	717	718	719
		[40	41	42]						
pCLL602	APP*	GTC	ATA	GCG	ACA	GTG	ATC	GTC	ATC	ACC
		V	I	A	T	V	I	V	I	T
pCLL611	717VI**	GTC	ATA	GCG	ACA	GTG	ATC	ATC	ATC	ACC
		V	I	A	T	V	I	I	I	T
pCLL612	717VG@	GTC	ATA	GCG	ACA	GTG	ATC	GGC	ATC	ACC
		V	I	A	T	V	I	G	I	T
pCLL613	717VF$	GTC	ATA	GCG	ACA	GTG	ATC	TTC	ATC	ACC
		V	I	A	T	V	I	F	I	T

TABLE 5 (continued)

DUTCH DISEASE			:	V (secretase clip)					
	686	687	:	688	689	690	691	692	693	694
	[15	16	:	17	18	19	20	21	22	23]
pCLL602 BAP*	CAA	AAA	:	TTG	GTG	TTC	TTT	GCG	GAA	GAT
	Q	K	:	L	V	F	F	A	E	D
pCLL603 BAP-22EQ#	CAA	AAA	:	TTG	GTG	TTC	TTT	GCA	CAA	GAT
pCLL606#	Q	K	:	L	V	F	F	A	Q	D

Notes:

\# APP-REP-751 and -770 derived BAP-22EQ constructs.

** Goate et al. (1991) Nature, 349:704-706; Yoshioka et al. (1991) BBRC 178:1141-1146; Naruse et al. (1991) Lancet 337:978-979.

@ Chartier-Harlin et al. (1991) Nature 353:844-846.

$ Murrell et al. (1991) Science 254:97-99.

Claims

1. A nucleic acid molecule encoding an amyloid precursor mutein, which comprises a nucleic acid sequence encoding at least one marker, the entire β-amyloid protein domain (BAP) or variants BAP_{E22Q} having an E to Q substitution at BAP amino acid #22 or $BAP_{\Delta aa11-28}$ and an amyloid precursor protein from which a block of 276 amino acids has been deleted.

2. The nucleic acid molecule of claim 1, wherein the nucleic acid molecule is a nucleic acid molecule selected from the group consisting of DNA, cDNA or RNA.

3. The nucleic acid molecule of claim 1, wherein the nucleic acid molecule is selected from the group consisting of pCLL964 (ATCC deposit no.: 68974) and pCLL602 (ATCC deposit no.: 68971).

4. The nucleic acid molecule of claim 1, wherein the amyloid precursor protein encoded by the nucleic acid sequence comprises 695, 751 or 770 amino acids prior to the deletion of the block of 276 amino acids.

5. A nucleic acid molecule encoding an amyloid precursor protein which comprises from the 5' end to the 3' end a nucleic acid sequence encoding a marker and an amyloid precursor protein comprising 695, 751 or 770 amino acids from which a block of 276 amino acids has been deleted, and which excludes the β-amyloid protein domain.

6. The nucleic acid molecule of claim 5, wherein the nucleic acid molecule is pCLL935 (ATCC deposit no.: 68972).

7. A nucleic acid molecule encoding an amyloid precursor protein which comprises from the 5' end to the 3' end a nucleic acid sequence encoding a marker and the β-amyloid protein domain variants BAP_{E22Q} having an E to Q substitution at BAP amino acid #22 or $BAP_{\Delta aa11\text{-}28}$.

8. A nucleic acid molecule encoding an amyloid precursor protein which comprises from the 5' end to the 3' end a nucleic acid sequence encoding Met-Enkephalin as a marker and the β-amyloid protein domain or variants BAP_{E22Q} having an E to Q substitution at BAP amino acid #22 or $BAP_{\Delta aa11\text{-}28}$

9. The nucleic acid molecule of claim 8, wherein the nucleic acid molecule is pCLL947 (ATCC deposit no.: 68973).

10. A vector comprising the nucleic acid molecule of claim 1, claim 5, claim 7 or claim 8.

11. A host cell stably transformed or transfected by a vector comprising the nucleic acid molecule of claim 1, claim 5, claim 7 or claim 8.

12. A recombinant polypeptide produced by the nucleic acid molecule of claim 1, claim 5, claim 7 or claim 8.

13. A method of detecting the presence of the recombinant polypeptide of claim 12 in a sample, comprising the steps of:

    (a) contacting an antibody directed to the marker and the sample under suitable conditions to favor the formation of an antibody-antigen complex, and
    (b) detecting the presence of any complex so formed.

14. A method of screening for a compound which inhibits or augments the formation of β-amyloid protein, comprising the steps of:

    (a) measuring the amount of marker in a suitable medium containing transfected cells stably or transiently expressing the nucleic acid molecule of claim 1,
    (b) treating said cells with the compound. and
    (c) testing the medium for an increase in the amount of the marker.

Patentansprüche

1. Nukleinsäuremolekül, das für ein Amyloidvorläufermutein kodiert, umfassend eine Nukleinsäuresequenz, die für mindestens einen Marker, für die gesamte β-Amyloidproteindomäne (BAP) oder deren Varianten BAP_{E22Q} mit einem E zu Q Austausch an BAP Aminosäure Nr. 22 oder $BAP_{\Delta aa11\text{-}28}$ und für ein Amyloidvorläuferprotein kodiert, von dem ein Block von 276 Aminosäuren deletiert wurde.

2. Das Nukleinsäuremolekül von Anspruch 1, das aus der Gruppe bestehend aus DNA, cDNA oder RNA ausgewählt ist.

3. Das Nukleinsäuremolekül von Anspruch 1, das aus der Gruppe bestehend aus pCLL964 (ATCC Hinterlegung Nr. 68974) und pCLL602 (ATCC Hinterlegung Nr. 68971) ausgewählt ist.

4. Das Nukleinsäuremolekül von Anspruch 1, wobei das von der Nukleinsäuresequenz kodierte Amyloidvorläuferprotein 695, 751 oder 770 Aminosäuren vor der Deletion des Blocks von 276 Aminosäuren umfasst.

5. Nukleinsäuremolekül, das für ein Amyloidvorläuferprotein kodiert, umfassend vom 5' zum 3' Ende eine Nukleinsäuresequenz, die für einen Marker und für ein Amyloidvorläuferprotein kodiert, das 695, 751 oder 770 Aminosäuren umfasst, von dem ein Block von 276 Aminosäuren deletiert wurde und das die β-Amyloidproteindomäne nicht umfasst.

6. Das Nukleinsäuremolekül von Anspruch 5, das pCLL935 (ATCC Hinterlegung Nr. 68972) ist.

7. Nukleinsäuremolekül, das für ein Amyloidvorläuferprotein kodiert, umfassend vom 5' zum 3' Ende eine Nukleinsäuresequenz, die für einen Marker und für die β-Amyioidproteindomän-Varianten BAP_{E22Q} mit einem E zu Q Austausch an BAP Aminosäure Nr. 22 oder $BAP_{\Delta aa11\text{-}28}$ kodiert.

8. Nukleinsäuremolekül, das für ein Amyloid-Vorläufer-Protein kodiert, umfassend vom 5' zum 3' Ende eine Nukleinsäuresequenz, die für Met-Enkephalin als Marker und für die β-Amyloidproteindomän-Varianten BAP_{E22Q} mit einem E zu Q Austausch an BAP Aminosäure Nr. 22 oder $BAP_{\Delta aa11-28}$ kodiert.

9. Das Nukleinsäuremolekül von Anspruch 8, das pCLL947 (ATCC Hinterlegung Nr. 68973) ist.

10. Vektor umfassend das Nukleinsäuremolekül von Anspruch 1, 5, 7 oder 8.

11. Wirtszelle, die mit einem Vektor umfassend das Nukleinsäuremolekül von Anspruch 1, 5, 7 oder 8 stabil transformiert oder transfiziert ist.

12. Rekombinantes Polypeptid, das mit dem Nukleinsäuremolekül von Anspruch 1, 5, 7 oder 8 hergestellt ist.

13. Verfahren zur Detektion der Anwesenheit des rekombinanten Polypeptids von Anspruch 12 in einer Probe, das die folgenden Schritte umfasst:

    (a) Kontaktieren eines gegen den Marker und die Probe gerichteten Antikörpers unter geeigneten Bedingungen, um die Bildung eines Antikörper-Antigen-Komplexes zu begünstigen, und
    (b) Detektieren eines so gebildeten Komplexes.

14. Verfahren zum Screening nach einer Verbindung, die die Bildung des β-Amyloidproteins inhibiert oder verstärkt, umfassend die Schritte:

    (a) Messen der Markermenge in einem geeigneten Medium, das transfizierte Zellen enthält, die stabil oder transient das Nukleinsäuremolekül von Anspruch 1 exprimieren,
    (b) Behandeln der Zellen mit der Verbindung und
    (c) Testen des Mediums auf eine Zunahme der Markermenge.

Revendications

1. Molécule d'acide nucléique codant pour une mutéine précurseur d'amyloïde, comprenant une séquence d'acide nucléique codant pour au moins un marqueur, le domaine complet de la protéine amyloïde β (BAP) ou le variant BAP_{E22Q} comportant un remplacement de E par Q à l'aminoacide N° 22 de la protéine BAP ou $BAP_{\Delta aa11-28,}$ et une protéine précurseur d'amyloïde dont un segment de 276 aminoacides a été supprimé.

2. Molécule d'acide nucléique selon la revendication 1, dans laquelle la molécule d'acide nucléique est une molécule d'acide nucléique choisie parmi un ADN, un ADNc ou un ARN.

3. Molécule d'acide nucléique selon la revendication 1, dans laquelle la molécule d'acide nucléique est choisie parmi pCLL964 (numéro de dépôt ATCC 68974) et pCLL602 (numéro de dépôt ATCC 68971).

4. Molécule d'acide nucléique selon la revendication 1, dans laquelle la protéine précurseur d'amyloïde codée par la séquence d'acide nucléique comprend 695, 751 ou 770 aminoacides avant la suppression du segment de 276 aminoacides.

5. Molécule d'acide nucléique codant pour une protéine précurseur d'amyloïde comprenant de l'extrémité 5' à l'extrémité 3', une séquence d'acide nucléique codant pour un marqueur et une protéine précurseur d'amyloïde comprenant 695, 751 ou 770 aminoacides dont un segment de 276 aminoacides a été supprimé, et qui exclut le domaine de la protéine amyloïde β.

6. Molécule d'acide nucléique selon la revendication 5, dans laquelle la molécule d'acide nucléique est pCLL935 (numéro de dépôt ATCC 68972).

7. Molécule d'acide nucléique codant pour une protéine précurseur d'amyloïde comprenant de l'extrémité 5' à l'extrémité 3', une séquence d'acide nucléique codant pour un marqueur et le variant de domaine de la protéine amyloïde β BAP_{E22Q} comportant un remplacement de E par Q à l'aminoacide N° 22 de la protéine BAP ou $BAP_{\Delta aa11-28}$.

8. Molécule d'acide nucléique codant pour une protéine précurseur d'amyloïde comprenant de l'extrémité 5' à l'extrémité 3', une séquence d'acide nucléique codant pour la mét-encéphaline en tant que marqueur, et le domaine de la protéine amyloïde β ou le variant BAP_{E22Q} comportant un remplacement de E par Q à l'aminoacide N° 22 de la protéine BAP, ou $BAP_{\Delta aa11-28}$.

9. Molécule d'acide nucléique selon la revendication 8, dans laquelle la molécule d'acide nucléique est pCLL947 (numéro de dépôt ATCC 68973).

10. Vecteur comprenant la molécule d'acide nucléique de la revendication 1, de la revendication 5, de la revendication 7 ou de la revendication 8.

11. Cellule hôte transformée ou transfectée de manière stable par un vecteur comprenant la molécule d'acide nucléique de la revendication 1, de la revendication 5, de la revendication 7 ou de la revendication 8.

12. Polypeptide recombinant produit par la molécule d'acide nucléique de la revendication 1, de la revendication 5, de la revendication 7 ou de la revendication 8.

13. Procédé de détection de la présence du polypeptide recombinant de la revendication 12 dans un échantillon, comprenant les étapes consistant :

(a) à mettre en contact un anticorps dirigé contre le marqueur et l'échantillon dans des conditions appropriées pour favoriser la formation d'un complexe anticorps-antigène, et
(b) à détecter la présence d'un complexe ainsi formé.

14. Procédé de sélection d'un composé empêchant ou augmentant la formation de la protéine amyloide β, comprenant les étapes consistant :

(a) à mesurer la quantité de marqueur dans un milieu approprié contenant des cellules transfectées de manière stable ou exprimant temporairement la molécule d'acide nucléique de la revendication 1,
(b) à traiter lesdites cellules avec le composé, et
(c) à tester le milieu à l'égard d'un accroissement de la quantité du marqueur.

Figure 1.

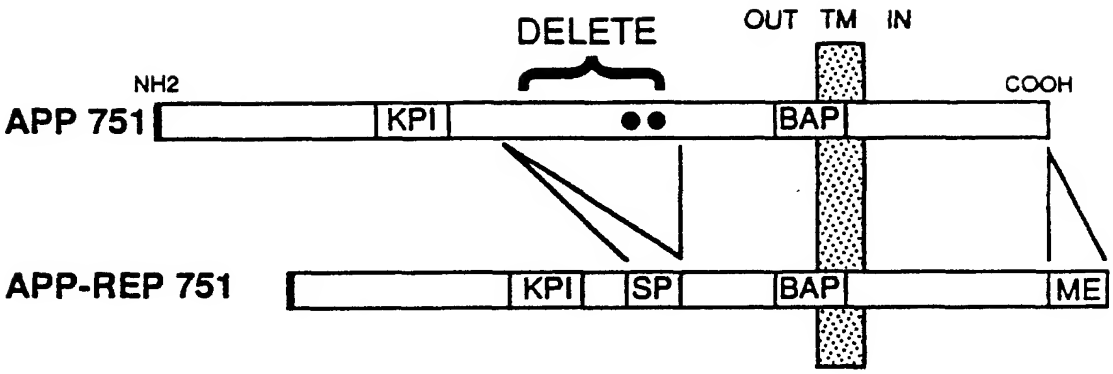

DELETE
OUT TM IN
NH2
COOH
APP 751
KPI
BAP
APP-REP 751
KPI
SP
BAP
ME

Figure 2.

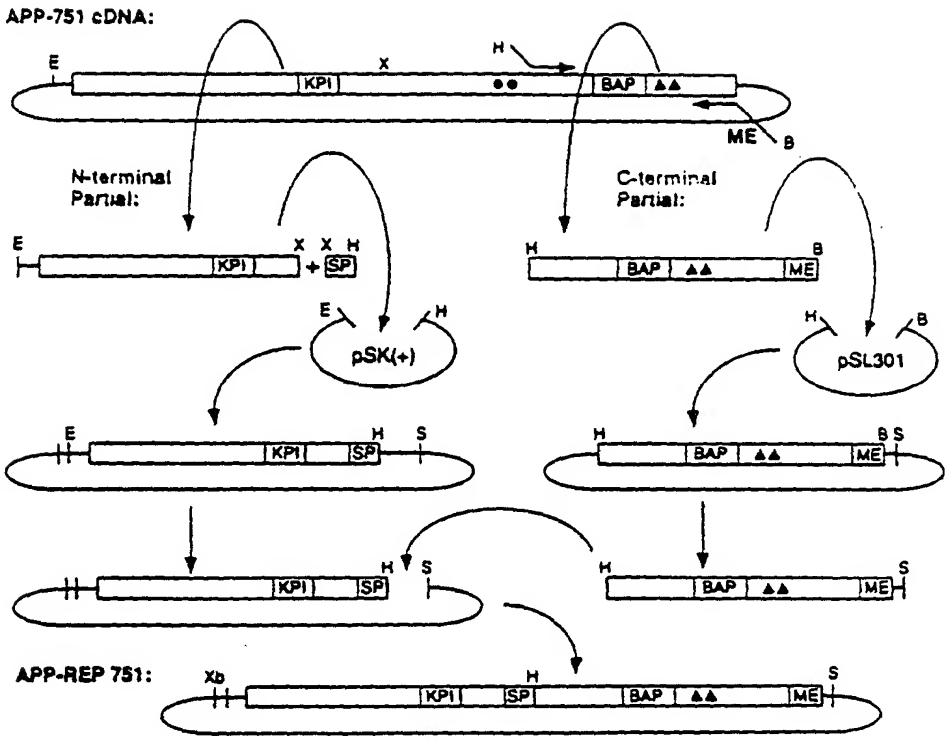

APP-751 cDNA:
E
X
H
KPI
BAP
ME
B
N-terminal Partial:
C-terminal Partial:
X X H
SP
pSK(+)
pSL301
S
APP-REP 751:
Xb

Figure 3.

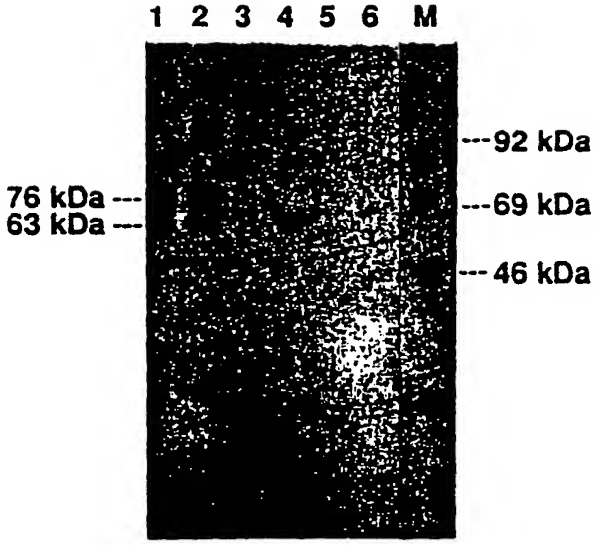


Figure 4.

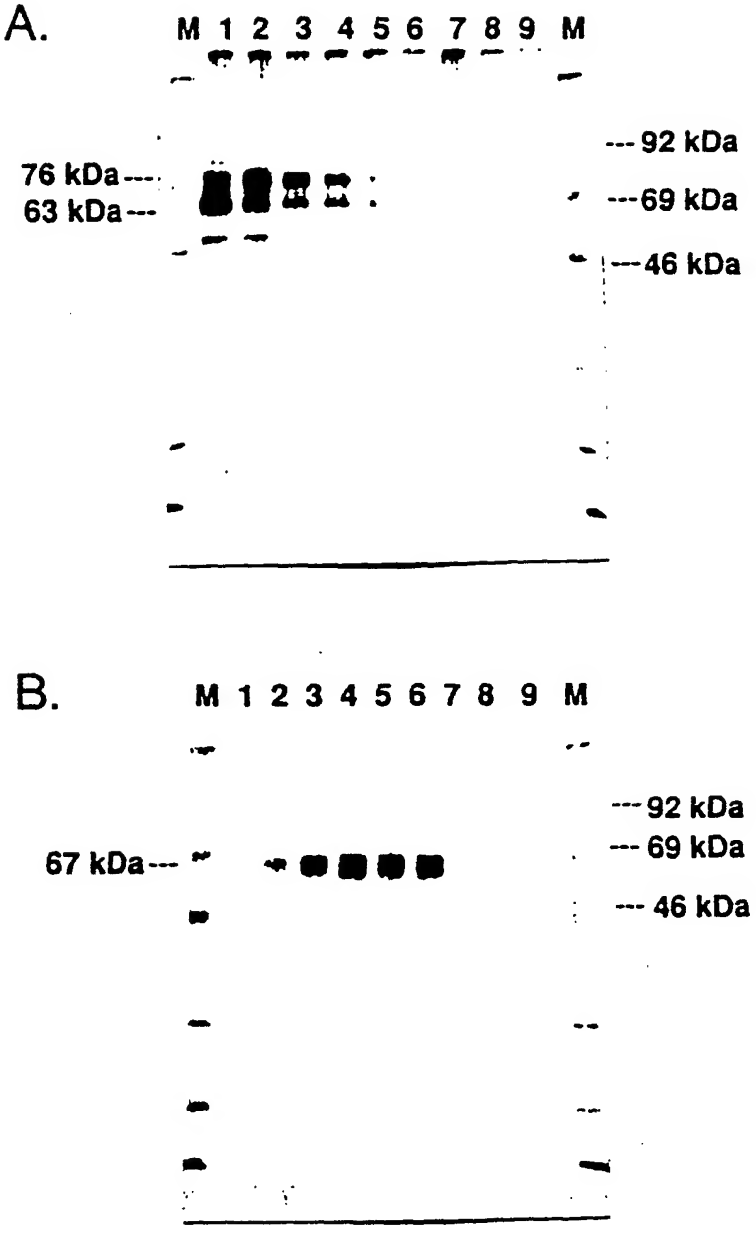
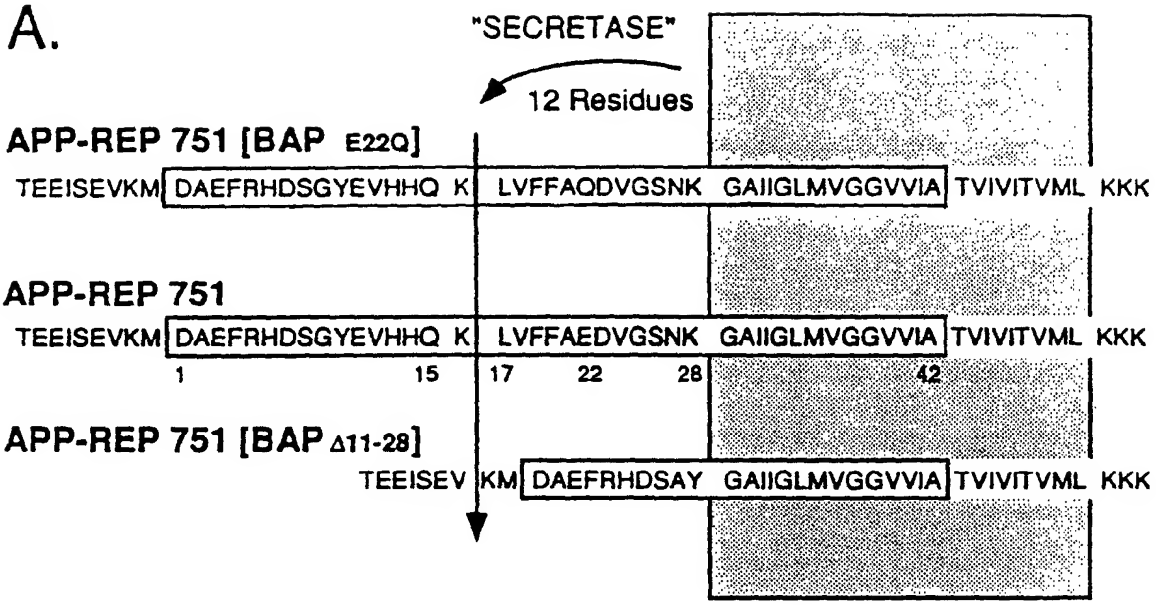

A.
M 1 2 3 4 5 6 7 8 9 M
76 kDa
63 kDa
92 kDa
69 kDa
46 kDa
B.
M 1 2 3 4 5 6 7 8 9 M
67 kDa
92 kDa
69 kDa
46 kDa

Figure 5.

A.


"SECRETASE"
12 Residues
APP-REP 751 [BAP E22Q]
TEEISEVKM DAEFRHDSGYEVHHQ K LVFFAQDVGSNK GAIIGLMVGGVVIA TVIVITVML KKK
APP-REP 751
TEEISEVKM DAEFRHDSGYEVHHQ K LVFFAEDVGSNK GAIIGLMVGGVVIA TVIVITVML KKK
1 15 17 22 28 42
APP-REP 751 [BAP Δ11-28]
TEEISEV KM DAEFRHDSAY GAIIGLMVGGVVIA TVIVITVML KKK

B.

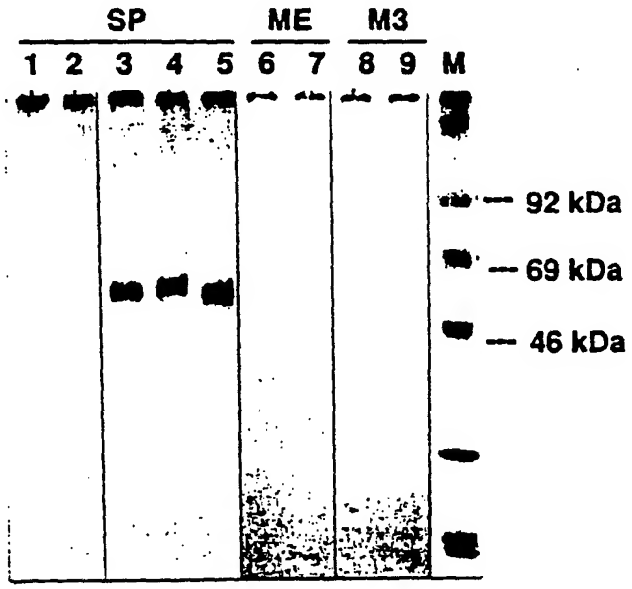
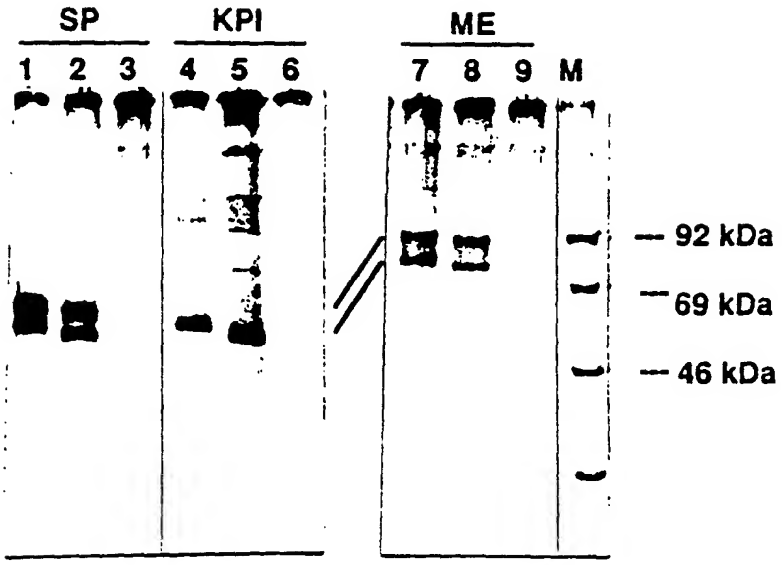

SP
ME
M3
1 2 3 4 5 6 7 8 9 M
92 kDa
69 kDa
46 kDa

Figure 5.

C.



D.

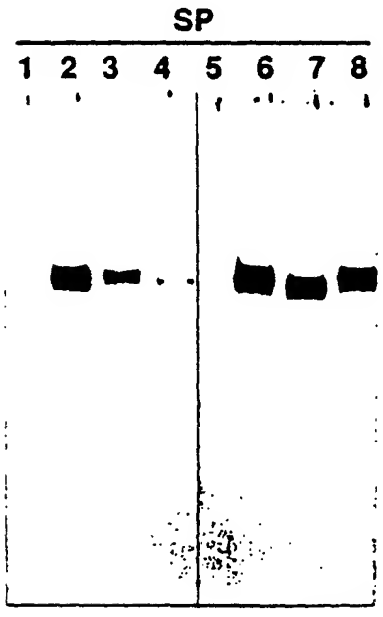


Figure 5.

E.

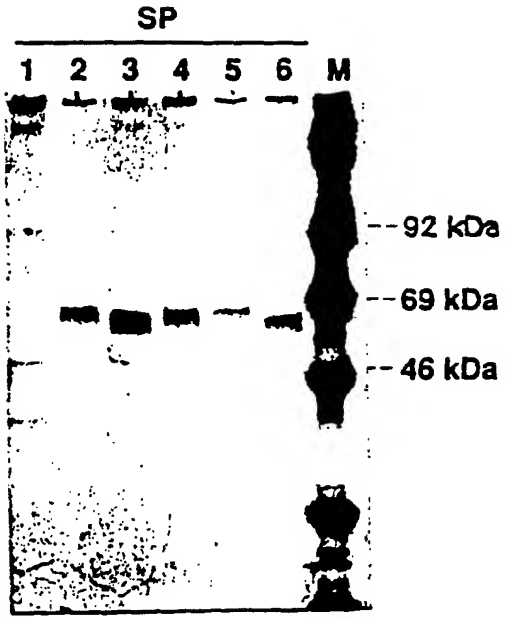


Figure 6.

A.

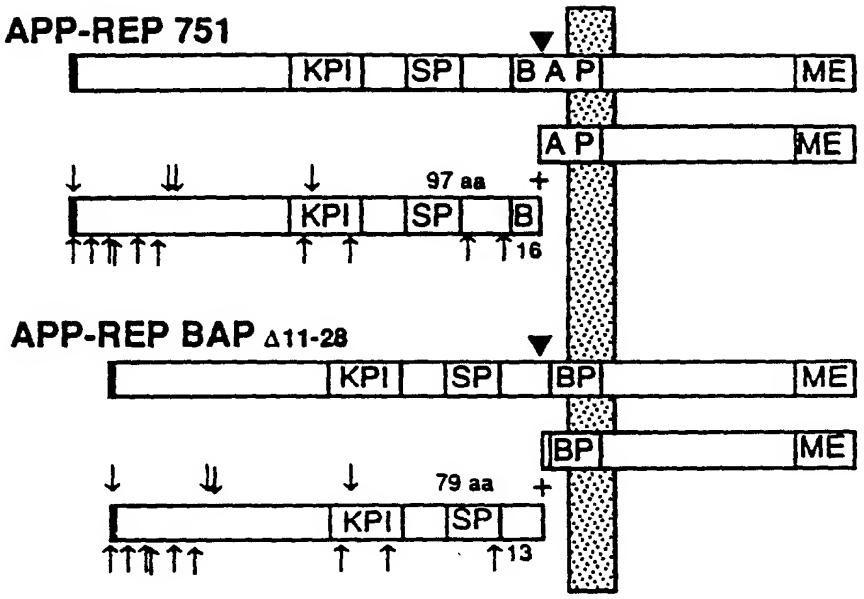
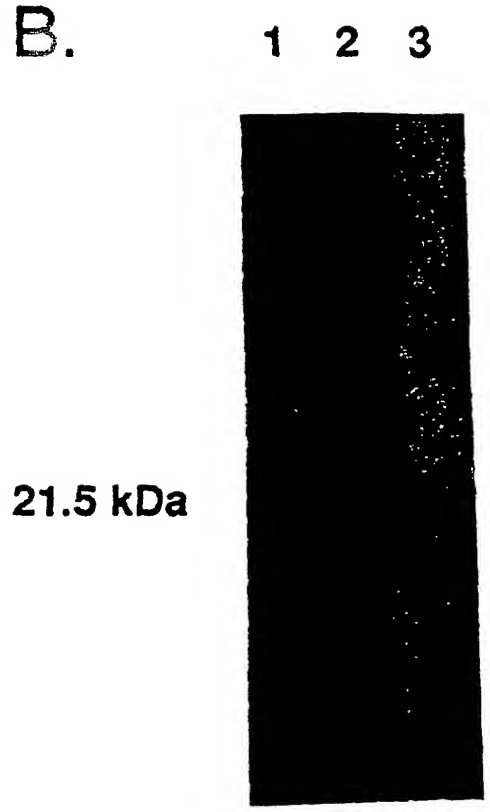

APP-REP 751
KPI
SP
B A P
ME
A P
ME
97 aa
KPI
SP
B
16
APP-REP BAP Δ11-28
KPI
SP
BP
ME
BP
ME
79 aa
KPI
SP
13

FIGURE 6.



```
SEQUENCE:  pCLL602  (APP-REP 751 protein)
VECTOR:    pcDNA-I-neo     (Invitrogen)
           pcDNA-I-neo-XS (JSJ modified polylinker to permit directional
              subcloning into XbaI-SalI sites)
INSERT:    XbaI-SalI fragment encoding APP-REP from pCLL964      16-1711
SEQUENCE:  5' polylinker:                                         2-47
              HindIII-XbaI from pcDNA-I-neo-XS                    2-15
              XbaI-EcoRI from pBluescript SK+                    16-47
           APP-REP 751:
              Amino-terminal partial from pCLL935):              48-1314
                 5' untranslated APP cDNA (from EcoRI)           48-195
                 N-terminal APP (to XhoI)                       196-1273
                 Substance P marker (XhoI to HindIII)          1274-1314
              Carboxy-terminal partial from pCLL947):          1314-1671
                 C-terminal APP and BAP (from novel HindIII)   1314-1656
                 Met-enkephalin marker (plus stop codon)       1657-1674
           3' polylinker:
              BamHI-SalI from pSL301                           1674-1711
              SalI-end of sequence from pcDNA-I-neo-XS         1712-1721


         10          20          30          40          50
          *           *           *           *           *
AAGCTTGGGG ATCCGCTCTA GAACTAGTGG ATCCCCCGGG CTGCAGGAAT
TTCGAACCCC TAGGCGAGAT CTTGATCACC TAGGGGGCCC GACGTCCTTA

         60          70          80          90         100
          *           *           *           *           *
TCGGGGGGGG CAGCGGTAGG CGAGAGCACG CGGAGGAGCG TGCGCGGGGC
AGCCCCCCCC GTCGCCATCC GCTCTCGTGC GCCTCCTCGC ACGCGCCCCG

        110         120         130         140         150
          *           *           *           *           *
CCCGGGAGAC GGCGGCGGTG GCGGCGCGGG CAGAGCAAGG ACGCGGCGGA
GGGCCCTCTG CCGCCGCCAC CGCCGCGCCC GTCTCGTTCC TGCGCCGCCT

        160         170         180         190
          *           *           *           *
TCCCACTCGC ACAGCAGCGC ACTCGGTGCC CCGCGCAGGG TCGCG
AGGGTGAGCG TGTCGTCGCG TGAGCCACGG GGCGCGTCCC AGCGC

   200         210         220         230         240
    *           *           *           *           *
ATG CTG CCC GGT TTG GCA CTG CTC CTG CTG GCC GCC TGG ACG GCT
TAC GAC GGG CCA AAC CGT GAC GAG GAC GAC CGG CGG ACC TGC CGA
Met Leu Pro Gly Leu Ala Leu Leu Leu Ala Ala Trp Thr Ala>

          250         260         270         280
           *           *           *           *
CGG GCG CTG GAG GTA CCC ACT GAT GGT AAT GCT GGC CTG CTG GCT GAA
GCC CGC GAC CTC CAT GGG TGA CTA CCA TTA CGA CCG GAC GAC CGA CTT
Arg Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu>
```

FIGURE 7

```
290          300          310          320          330
  *            *            *            *            *
CCC CAG ATT GCC ATG TTC TGT GGC AGA CTG AAC ATG CAC ATG AAT GTC
GGG GTC TAA CGG TAC AAG ACA CCG TCT GAC TTG TAC GTG TAC TTA CAG
Pro Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val>

  340          350          360          370          380
    *            *            *            *            *
CAG AAT GGG AAG TGG GAT TCA GAT CCA TCA GGG ACC AAA ACC TGC ATT
GTC TTA CCC TTC ACC CTA AGT CTA GGT AGT CCC TGG TTT TGG ACG TAA
Gln Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile>

    390          400          410          420          430
      *            *            *            *            *
GAT ACC AAG GAA GGC ATC CTG CAG TAT TGC CAA GAA GTC TAC CCT GAA
CTA TGG TTC CTT CCG TAG GAC GTC ATA ACG GTT CTT CAG ATG GGA CTT
Asp Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu>

      440          450          460          470          480
        *            *            *            *            *
CTG CAG ATC ACC AAT GTG GTA GAA GCC AAC CAA CCA GTG ACC ATC CAG
GAC GTC TAG TGG TTA CAC CAT CTT CGG TTG GTT GGT CAC TGG TAG GTC
Leu Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln>

          490          500          510          520
            *            *            *            *
AAC TGG TGC AAG CGG GGC CGC AAG CAG TGC AAG ACC CAT CCC CAC TTT
TTG ACC ACG TTC GCC CCG GCG TTC GTC ACG TTC TGG GTA GGG GTG AAA
Asn Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe>

530          540          550          560          570
  *            *            *            *            *
GTG ATT CCC TAC CGC TGC TTA GTT GGT GAG TTT GTA AGT GAT GCC CTT
CAC TAA GGG ATG GCG ACG AAT CAA CCA CTC AAA CAT TCA CTA CGG GAA
Val Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu>

  580          590          600          610          620
    *            *            *            *            *
CTC GTT CCT GAC AAG TGC AAA TTC TTA CAC CAG GAG AGG ATG GAT GTT
GAG CAA GGA CTG TTC ACG TTT AAG AAT GTG GTC CTC TCC TAC CTA CAA
Leu Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val>

    630          640          650          660          670
      *            *            *            *            *
TGC GAA ACT CAT CTT CAC TGG CAC ACC GTC GCC AAA GAG ACA TGC AGT
ACG CTT TGA GTA GAA GTG ACC GTG TGG CAG CGG TTT CTC TGT ACG TCA
Cys Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser>

      680          690          700          710          720
        *            *            *            *            *
GAG AAG AGT ACC AAC TTG CAT GAC TAC GGC ATG TTG CTG CCC TGC GGA
CTC TTC TCA TGG TTG AAC GTA CTG ATG CCG TAC AAC GAC GGG ACG CCT
Glu Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly>
```

FIGURE 7
(continued)

```
          730         740         750         760
           *           *           *           *
ATT GAC AAG TTC CGA GGG GTA GAG TTT GTG TGT TGC CCA CTC CCT GAA
TAA CTG TTC AAG GCT CCC CAT CTC AAA CAC ACA ACG TGT GAC CGA CTT
Ile Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu>

770         780         790         800         810
 *           *           *           *           *
GAA AGT GAC AAT GTG GAT TCT GCT GAT GCG GAG GAG GAT GAC TCG GAT
CTT TCA CTG TTA CAC CTA AGA CGA CTA CGC CTC CTC CTA CTG AGC CTA
Glu Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp>

  820         830         840         850         860
   *           *           *           *           *
GTC TGG TGG GGC GGA GCA GAC ACA GAC TAT GCA GAT GGG AGT GAA GAC
CAG ACC ACC CCG CCT CGT CTG TGT CTG ATA CGT CTA CCC TCA CTT CTG
Val Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp>

    870         880         890         900         910
     *           *           *           *           *
AAA GTA GTA GAA GTA GCA GAG GAG GAA GAA GTG GCT GAG GTG GAA GAA
TTT CAT CAT CTT CAT CGT CTC CTC CTT CTT CAC CGA CTC CAC CTT CTT
Lys Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu>

       920         930         940         950         960
        *           *           *           *           *
GAA GAA GCC GAT GAT GAC GAG GAC GAT GAG GAT GGT GAT GAG GTA GAG
CTT CTT CGG CTA CTA CTG CTC CTG CTA CTC CTA CCA CTA CTC CAT CTC
Glu Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu>

          970         980         990         1000
           *           *           *           *
GAA GAG GCT GAG GAA CCC TAC GAA GAA GCC ACA GAG AGA ACC ACC AGC
CTT CTC CGA CTC CTT GGG ATG CTT CTT CGG TGT CTC TCT TGG TGG TCG
Glu Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser>

1010        1020        1030        1040        1050
  *           *           *           *           *
ATT GCC ACC ACC ACC ACC ACC ACC ACA GAG TCT GTG GAA GAG GTG GTT
TAA CGG TGG TGG TGG TGG TGG TGG TGT CTC AGA CAC CTT CTC CAC CAA
Ile Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val>

  1060        1070        1080        1090        1100
   *           *           *           *           *
CGA GAG GTG TGC TCT GAA CAA GCC GAG ACG GGG CCG TGC CGA GCA ATG
GCT CTC CAC ACG AGA CTT GTT CGG CTC TGC CCC GGC ACG GCT CGT TAC
Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met>

    1110        1120        1130        1140        1150
     *           *           *           *           *
ATC TCC CGC TGG TAC TTT GAT GTG ACT GAA GGG AAG TGT GCC CCA TTC
TAG AGG GCG ACC ATG AAA CTA CAC TGA CTT CCC TTC ACA CGG GGT AAG
Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe>
```

FIGURE 7
(continued)

```
      1160        1170        1180        1190        1200
        *           *           *           *           *
TTT TAC GGC GGA TGT GGC GGC AAC CGG AAC AAC TTT GAC ACA GAA GAG
AAA ATG CCG CCT ACA CCG CCG TTG GCC TTG TTG AAA CTG TGT CTT CTC
Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu>

         1210        1220        1230        1240
           *           *           *           *
TAC TGC ATG GCC GTG TGT GGC AGC GCC ATT CCT ACA ACA GCA GCC AGT
ATG ACG TAC CGG CAC ACA CCG TCG CGG TAA GGA TGT TGT CGT CGG TCA
Tyr Cys Met Ala Val Cys Gly Ser Ala Ile Pro Thr Thr Ala Ala Ser>

1250        1260        1270        1280        1290
  *           *           *           *           *
ACC CCT GAT GCC GTT GAC AAG TAT CTC GAG CGG CCC AAG CCC CAG CAG
TGG GGA CTA CGG CAA CTG TTC ATA GAG CTC GCC GGG TTC GGG GTC GTC
Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Arg Pro Lys Pro Gln Gln>

 1300        1310        1320        1330        1340
   *           *           *           *           *
TTC TTT GGC CTG ATG GGA AGC TTG ACA AAT ATC AAG ACG GAG GAG ATC
AAG AAA CCG GAC TAC CCT TCG AAC TGT TTA TAG TTC TGC CTC CTC TAG
Phe Phe Gly Leu Met Gly Ser Leu Thr Asn Ile Lys Thr Glu Glu Ile>

   1350        1360        1370        1380        1390
     *           *           *           *           *
TCT GAA GTG AAG ATG GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA
AGA CTT CAC TTC TAC CTA CGT CTT AAG GCT GTA CTG AGT CCT ATA CTT
Ser Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu>

      1400        1410        1420        1430        1440
        *           *           *           *           *
GTT CAT CAT CAA AAA TTG GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC
CAA GTA GTA GTT TTT AAC CAC AAG AAA CGT CTT CTA CAC CCA AGT TTG
Val His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn>

         1450        1460        1470        1480
           *           *           *           *
AAA GGT GCA ATC ATT GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG ACA
TTT CCA CGT TAG TAA CCT GAG TAC CAC CCG CCA CAA CAG TAT CGC TGT
Lys Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr>

1490        1500        1510        1520        1530
  *           *           *           *           *
GTG ATC GTC ATC ACC TTG GTG ATG CTG AAG AAG AAA CAG TAC ACA TCC
CAC TAG CAG TAG TGG AAC CAC TAC GAC TTC TTC TTT GTC ATG TGT AGG
Val Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser>

 1540        1550        1560        1570        1580
   *           *           *           *           *
ATT CAT CAT GGT GTG GTG GAG GTT GAC GCC GCT GTC ACC CCA GAG GAG
TAA GTA GTA CCA CAC CAC CTC CAA CTG CGG CGA CAG TGG GGT CTC CTC
Ile His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu>
```

FIGURE 7
(continued)

```
     30          1600         1610         1620          1630
      *            *            *            *             *
CGC CAC CTG TCC AAG ATG CAG CAG AAC GGC TAC GAA AAT CCA ACC TAC
GCG GTG GAC AGG TTC TAC GTC GTC TTG CCG ATG CTT TTA GGT TGG AAG
Arg His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr>

     1640         1650         1660         1670         1680
        *            *            *            *            *
AAG TTC TTT GAG CAG ATG CAG AAC TAT GGG GGC TTC ATG TAG GATCCA
TTC AAG AAA CTC GTC TAC GTC TTG ATA CCC CCG AAG TAC ATC CTAGGT
Lys Phe Phe Glu Gln Met Gln Asn Tyr Gly Gly Phe Met ***

     1690      1700      1710        1720
        *         *         *           *
TATATAGGGC CCGGGTTAT AATTACCTCA GGTCGACCTA GA
ATATATCCCG GGCCCAATA TTAATGGAGT CCAGCTGGAT CT
```

FIGURE 7
(continued)

```
Total of 12 files.
 RMRD3>TYPE APP770.
FOCUS       HUMAFPA4     3353 bp ss-mRNA            PRI       15-JUN-1989
Human amyloid A4 mRNA, complete cds.
ACCESSION   Y00264
KEYWORDS    amyloid fibril protein; cell surface glycoprotein.
SOURCE      human (Homo sapiens).
  ORGANISM  Homo sapiens
            Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia;
            Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae.
REFERENCE   1  (bases 1 to 3353; enum. -146 to 3207, no zero)
  AUTHORS   Kang,J., Lemaire,H.G., Unterbeck,A., Salbaum,J.M., Masters,C.L.,
            Grzeschik,K.-H., Multhaup,G., Beyreuther,K. and Mueller-Hill,B.
  TITLE     The precursor of Alzheimer's disease amyloid A4 protein resembles a
            cell-surface receptor
  JOURNAL   Nature 325, 733-736 (1987)
  STANDARD  simple automatic
REFERENCE   2  (bases 1 to 3353; enum. 1 to 3353)
  AUTHORS   Mueller Hill,B.
  JOURNAL   Unpublished (1987) Submitted to the EMBL data library.
  STANDARD  simple automatic
COMMENT     *source: tissue=cortex of brain; *source: developmental
            stage=5-month-old aborted fetus;
               EMBL features not translated to GenBank features:
               key        from     to       description
               SITE       3080     3085     polyA signal
               SITE       3089     3094     polyA signal
               SITE       3331     3336     polyA signal
               POLYA      3353     3353     polyA site
FEATURES    from  to/span     description
    pept     147     2234     amyloid A4 /nomgen="APP" /map="21q21.2"
                              /hgml_locus_uid="LG0136J"
BASE COUNT    922 a    745 c    867 g    819 t
ORIGIN

      App770.  Length: 3353  May 29, 1990  11:30  Check: 6510  ..

        1  AGTTTCCTCG GCAGCGGTAG GCGAGAGCAC GCGGAGGAGC GTGCGCGGGG

       51  CCCCGGGAGA CGGCGGCGGT GGCGGCGCGG GCAGAGCAAG GACGCGGCGG

      101  ATCCCACTCG CACAGCAGCG CACTCGGTGC CCCGCGCAGG GTCGCGATGC

      151  TGCCCGGTTT GGCACTGCTC CTGCTGGCCG CCTGGACGGC TCGGGCGCTG

      201  GAGGTACCCA CTGATGGTAA TGCTGGCCTG CTGGCTGAAC CCCAGATTGC

      251  CATGTTCTGT GGCAGACTGA ACATGCACAT GAATGTCCAG AATGGGAAGT

      301  GGGATTCAGA TCCATCAGGG ACCAAAACCT GCATTGATAC CAAGGAAGGC

      351  ATCCTGCAGT ATTGCCAAGA AGTCTACCCT GAACTGCAGA TCACCAATGT

      401  GGTAGAAGCC AACCAACCAG TGACCATCCA GAACTGGTGC AAGCGGGGCC

      451  GCAAGCAGTG CAAGACCCAT CCCCACTTTG TGATTCCCTA CCGCTGCTTA

      501  GTTGGTGAGT TTGTAAGTGA TGCCCTTCTC GTTCCTGACA AGTGCAAATT

      551  CTTACACCAG GAGAGGATGG ATGTTTGCGA AACTCATCTT CACTGGCACA

      601  CCGTCGCCAA AGAGACATGC AGTGAGAAGA GTACCAACTT GCATGACTAC

      651  GGCATGTTGC TGCCCTGCGG AATTGACAAG TTCCGAGGGG TAGAGTTTGT

      701  GTGTTGCCCA CTGGCTGAAG AAAGTGACAA TGTGGATTCT GCTGATGCGG
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FIGURE 8

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 751  AGGAGGATGA CTCGGATGTC TGGTGGGGCG GAGCAGACAC AGA TATGCA
 801  GATGGGAGTG AAGACAAAGT AGTAGAAGTA GCAGAGGAGG AAGAAGTGGC
 851  TGAGGTGGAA GAAGAAGAAG CCGATGATGA CGAGGACGAT GAGGATGGTG
 901  ATGAGGTAGA GGAAGAGGCT GAGGAACCCT ACGAAGAAGC CACAGAGAGA
 951  ACCACCAGCA TTGCCACCAC CACCACCACC ACCACAGAGT CTGTGGAAGA
1001  GGTGGTTCGA GTTCCTACAA CAGCAGCCAG TACCCCTGAT GCCGTTGACA
1051  AGTATCTCGA GACACCTGGG GATGAGAATG AACATGCCCA TTTCCAGAAA
1101  GCCAAAGAGA GGCTTGAGGC CAAGCACCGA GAGAGAATGT CCCAGGTCAT
1151  GAGAGAATGG GAAGAGGCAG AACGTCAAGC AAAGAACTTG CCTAAAGCTG
1201  ATAAGAAGGC AGTTATCCAG CATTTCCAGG AGAAAGTGGA ATCTTTGGAA
1251  CAGGAAGCAG CCAACGAGAG ACAGCAGCTG GTGGAGACAC ACATGGCCAG
1301  AGTGGAAGCC ATGCTCAATG ACCGCCGCCG CCTGGCCCTG GAGAACTACA
1351  TCACCGCTCT GCAGGCTGTT CCTCCTCGGC CTCGTCACGT GTTCAATATG
1401  CTAAAGAAGT ATGTCCGCGC AGAACAGAAG GACAGACAGC ACACCCTAAA
1451  GCATTTCGAG CATGTGCGCA TGGTGGATCC CAAGAAAGCC GCTCAGATCC
1501  GGTCCCAGGT TATGACACAC CTCCGTGTGA TTTATGAGCG CATGAATCAG
1551  TCTCTCTCCC TGCTCTACAA CGTGCCTGCA GTGGCCGAGG AGATTCAGGA
1601  TGAAGTTGAT GAGCTGCTTC AGAAAGAGCA AAACTATTCA GATGACGTCT
1651  TGGCCAACAT GATTAGTGAA CCAAGGATCA GTTACGGAAA CGATGCTCTC
1701  ATGCCATCTT TGACCGAAAC GAAAACCACC GTGGAGCTCC TTCCCGTGAA
1751  TGGAGAGTTC AGCCTGGACG ATCTCCAGCC GTGGCATTCT TTTGGGGCTG
1801  ACTCTGTGCC AGCCAACACA GAAAACGAAG TTGAGCCTGT TGATGCCCGC
1851  CCTGCTGCCG ACCGAGGACT GACCACTCGA CCAGGTTCTG GGTTGACAAA
1901  TATCAAGACG GAGGAGATCT CTGAAGTGAA GATGGATGCA GAATTCCGAC
1951  ATGACTCAGG ATATGAAGTT CATCATCAAA AATTGGTGTT CTTTGCAGAA
2001  GATGTGGGTT CAAACAAAGG TGCAATCATT GGACTCATGG TGGGCGGTGT
2051  TGTCATAGCG ACAGTGATCG TCATCACCTT GGTGATGCTG AAGAAGAAAC
2101  AGTACACATC CATTCATCAT GGTGTGGTGG AGGTTGACGC CGCTGTCACC
2151  CCAGAGGAGC GCCACCTGTC CAAGATGCAG CAGAACGGCT ACGAAAATCC
2201  AACCTACAAG TTCTTTGAGC AGATGCAGAA CT

                                        AGACCCCC GCCACAGCAG
2251  CCTCTGAAGT TGGACAGCAA AACCATTGCT TCACTACCCA TCGGTGTCCA
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FIGURE 8
(continued)

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2300  TTTATAGAAT AATGTGCGAA GAAACAAACC CGTTTTATGA TTTACTCATT
2351  ATCGCCTTTT GACAGCTGTG CTGTAACACA AGTACATGCC TGAACTTGAA
2401  TTAATCCACA CATCAGTAAT GTATTCTATC TCTCTTTACA TTTTGGTCTC
2451  TATACTACAT TATTAATGGG TTTTGTGTAC TGTAAAGAAT TTAGCTGTAT
2501  CAAACTAGTG CATGAATAGA TTCTCTCCTG ATTATTTATC ACATAGCCCC
2551  TTAGCCAGTT GTATATTATT CTTGTGGTTT GTGACCCAAT TAAGTCCTAC
2601  TTTACATATG CTTTAAGAAT CGATGGGGGA TGCTTCATGT GAACGTGGGA
2651  GTTCAGCTGC TTCTCTTGCC TAAGTATTCC TTTCCTGATC ACTATGCATT
2701  TTAAAGTTAA ACATTTTTAA GTATTTCAGA TGCTTTAGAG AGATTTTTTT
2751  TCCATGACTG CATTTTACTG TACAGATTGC TGCTTCTGCT ATATTTGTGA
2801  TATAGGAATT AAGAGGATAC ACACGTTTGT TTCTTCGTGC CTGTTTTATG
2851  TGCACACATT AGGCATTGAG ACTTCAAGCT TTTCTTTTTT TGTCCACGTA
2901  TCTTTGGGTC TTTGATAAAG AAAAGAATCC CTGTTCATTG TAAGCACTTT
2951  TACGGGGCGG GTGGGGAGGG GTGCTCTGCT GGTCTTCAAT TACCAAGAAT
3001  TCTCCAAAAC AATTTTCTGC AGGATGATTG TACAGAATCA TTGCTTATGA
3051  CATGATCGCT TTCTACACTG TATTACATAA ATAAATTAAA TAAAATAACC
3101  CCGGGCAAGA CTTTTCTTTG AAGGATGACT ACAGACATTA AATAATCGAA
3151  GTAATTTTGG GTGGGGAGAA GAGGCAGATT CAATTTTCTT TAACCAGTCT
3201  GAAGTTTCAT TTATGATACA AAAGAAGATG AAAATGGAAG TGGCAATATA
3251  AGGGGATGAG GAAGGCATGC CTGGACAAAC CCTTCTTTTA AGATGTGTCT
3301  TCAATTTGTA TAAAATGGTG TTTTCATGTA AATAAATACA TTCTTGGAGG
3351  AGC
RMRD3>
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FIGURE 8
(continued)